Laat niets of niemand je denken fnuiken
(Gebod VIII)
Durf Denken campagne UGent

Promotor: **Prof. dr. ir. Mieke Uyttendaele**
Department of Food Safety and Food Quality
Faculty of Bioscience Engineering, Ghent University

Dean: **Prof. dr. ir. Guido Van Huylenbroeck**

Rector: **Prof. dr. Anne De Paepe**

Examination Committee Chairman: **Prof. dr. ir. Guy Smagghe**
Examination Committee Secretary: **Prof. dr. Imca Sampers**
Examination Committee: **Prof. dr. ir. Mieke Uyttendaele** (UGent), **dr. Andreja Rajkovic** (UGent), **Prof. dr. Lieven De Zutter** (UGent), **dr. ir. Sarah Denayer** (WIV), **dr. Erwin Duizer** (RIVW, NL), **dr. Paul Cook** (FSA, UK)

ir. Ann De Keuckelaere

Detection and control of noroviruses in fresh produce

Thesis submitted in fulfillment of the requirements for the degree of
Doctor (PhD) in Applied Biological Sciences

Dutch translation of the title:

Detectie en controle van norovirussen in groenten en fruit

Illustration cover: ©iStock.com/AnnDeKeuckelaere

To refer to this thesis:

De Keuckelaere, A. (2015). Detection and control of noroviruses in fresh produce. PhD thesis, Ghent University, Belgium.

ISBN-number: 978-90-5989-778-6

WOORD VAN DANK

Dit norovirus verhaal begon 6 jaar geleden, in het eerste jaar van mijn master, met de keuze van het onderwerp 'Detectie van norovirus in levensmiddelen en water' als eindwerk. Ik had nooit gedacht dat deze keuze mijn leven de komende vijf en een half jaar zou gaan beïnvloeden. Gedurende het eerste jaar thesis, gevolgd door 4,5 jaar onderzoek werd ik intens begeleid en gesteund door een heleboel mensen. Tot hen wil ik een woordje van dank richten.

Mijn oprechte dank gaat uit naar mijn promotor prof. Mieke Uyttendaele voor de kans om in het Veg-i-Trade project te stappen en voor haar verruimend perspectief, kritische zin en steun gedurende het hele onderzoek en het aansluitend schrijfwerk. Verder wil ik mijn begeleiders bedanken: dr. Ambroos Stals, wie mij enthousiast maakte over het onderwerp en introduceerde in RT-qPCR, voor de steun tijdens de labo experimenten en het vele schrijfwerk; dr. Leen Baert voor haar gedrevenheid, haar steun en begeleiding voor het behalen van een IWT doctoraatsbeurs en gedurende de water-experimenten; I would also like to express my gratitude to dr. Dan Li for the many discussions and her effort to structure, correct and contain this PhD literature study and discussion.

Naast mijn begeleiders (het 'virus team') wil ik ook al mijn coauteurs bedanken voor hun bijdrage en het nalezen van het manuscript, hierbij in het bijzonder Kevin voor de vlotte samenwerking aan hoofdstuk 5 en prof. Liesbeth Jacxsens voor de intense begeleiding tijdens het doorploegen van alle QMRA artikels voor de review in hoofdstuk 6. Hiernaast gaat mijn dank ook uit naar dr. John Donaghy (Nestlé) voor zijn nazicht van hoofdstuk 4, naar prof. Lee-Ann Jaykus voor het grondig herformuleren van hoofdstuk 6, en naar dr. Siele Ceuppens voor het nalezen van hoofdstuk 1 en 7. Tevens werd deze bundeling van onderzoeksresultaten nauwgezet gelezen door de leden van de examencommissie. De geleverde commentaren en suggesties hebben een aanzienlijke bijdrage geleverd tot dit proefschrift, waarvoor dank.

Voor het experimentele luik heb ik hulp gekregen van diverse mensen buiten het 'virus team'. As such I would like to thank Alexandra for her optimism, and practical insight and help during the water experiments (Chapter 2). Ook een dikke merci aan Elien voor haar inzet, hulp en gezelschap tijdens de analyses voor het Veg-i-Trade project en de labo experimenten die hebben geleid tot hoofdstuk 4 en 5. Ook aan Markus voor het bijspringen tijdens de hectische was-experimenten (hoofdstuk 5). Tijdens deze periode aan het LFMFP- labo heb ik tevens het geluk gehad om Bart als thesisstudent te mogen begeleiden. Na veel zweten op de detectiemethode en onverklaarbare lage recovery's

leidde dit werk uiteindelijk tot hoofdstuk 4 en nieuwe inzichten omtrent het belang van de kwaliteit van het proces controle virus.

Ook mijn collega's van het LFMFP wens ik te bedanken voor de samenwerking en de vlotte babbel. Also special thanks to my fellow 'outcasts' to block C: the 'virus team', Alexandra, Siele and Sigrid, of whom Dan is the only virus team member that knew to escape from Block C and its terrible view. Thanks to them, moments of intense work could be interchanged with moments of diversion and laughter.

En ten slotte, bedankt aan mijn ouders, zus en Maarten. Voor mijn ouders die mij steeds gesteund en gestimuleerd hebben om het beste van mijzelf te geven en zonder wie ik nooit zou staan waar ik nu sta. Voor wie helpen een evidentie is en wie steeds klaarstaan om met mij de volgende uitdaging aan te gaan. Voor Maarten om mij tijdens deze rush naar het einde toe te steunen en om actief het voortouw te nemen in ons bouwproject en zoveel meer. Woorden schieten te kort om hen voldoende te bedanken.

Ann

Borgerhout, februari 2015

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AdV	adenovirus
AFNOR	Association française de Normalisation
AGE	acute gastroenteritis
AGWR	Australian guidelines for water recycling
ANOVA	analysis of variance
ATCC	American type culture collection (United States of America)
approx.	approximately
BHI	Brain heart infusion broth
bPyV	bovine polyomavirus
CAC	Codex Alimenterius Commission
CaCV	canine calicivirus
C:B	chloroform:butanol
CC	cell-culture
CDC	Centers for Disease Control and Prevention
cDNA	copy deoxyribonucleic acid
CEN	Comité Européen de Normalisation (European Committee for Standardization)
CFU	colony-forming unit
CI	confidence interval
COD	chemical oxygen demand
Ct	cycle threshold
DALY	disability adjusted life-years
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
EC	European Commission
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
EIA	enzyme immunoassay
EM	electron microscopy
EPA	Environmental Protection Agency
EU	European Union
FAO	Food and Agricultural Organization
FAVV	Federaal Agentschap voor de veiligheid van de voedselketen
FBO	foodborne outbreak
FBVE	Food Borne Virus European network
FC	free chlorine
FCV	feline calicivirus
FDA	Food and Drug Administration
FH	food handler
FoAO	food of animal origin
FoNAO	food of non-animal origin
G	genogroup (GI: genogroup 1, GII: genogroup 2)

GAP	good agricultural practices
GC	genomic copies
GHP	good hygienic practices
GL	gloves
GSE	grape seed extract
HACCP	Hazard Analysis and Critical Control Points
hAdV	human adenovirus
HAV	hepatitis A virus
HBGA	histo-blood group antigen
HD-PE	high-density polyethylene
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPP	high pressure processing
IAC	internal amplification control
Ig	immunoglobulin
Ip	isoelectric point
IPC	internal amplification control
IQF	individual quick frozen
ISO	International Organization for Standardization
KW	Kruskal-Wallis (test)
LFMFP	Laboratory for Food Microbiology and Food Preservation (Ghent University, Belgium)
(p)LOD	(practical) limit of detection
LOQ	limit of quantification
LP	low-pressure
MAP	modified atmosphere packaging
MNV-1	Murine norovirus 1
MOI	multiplicity of infection
MRA	microbial risk assessment
MW	Mann-Whitney (test)
N_{50}	microbial dose eliciting 50% infections in the exposed population
Nal^R	nalidixic acid-resistant
NHANES	US National Health and Nutrition Examination Survey
NLV	Norwalk-like viruses
NoV	norovirus
NR	neoprene rubber
NV	Norwalk virus
OC	operating characteristic
PAA	peroxyacetic acid
pAdV	porcine adenovirus
PBS	phosphate buffered saline
PC	process control
PEG	polyethyleenglycol
PFU	plaque forming unit
PGM-MB	porcine gastric mucin binding magnetic bead

PL	pulsed light
pppy	per person per year
PV	poliovirus
QMRA	quantitative microbial risk assessment
RASFF	rapid alert system for food and feed
RdRp	RNA dependent RNA polymerase
RH	relative humidity
RIVM	Rijksinstituut voor Volksgezondheid en Milieu
RNA	ribonucleic acid
RT	room temperature
RT-PCR(U)	reverse transcription polymerase chain reaction (units)
RTC	reverse transcription control
RTE	ready-to-eat
RT-qPCR	real-time reverse transcription polymerase chain reaction
RV	rotavirus
SD	standard deviation
SEP	standard error of prediction
spp.	species
SRE	sample recovery efficiency
SRSVs	small, round structured viruses
ss	single stranded
$TCID_{50}$	median tissue culture infectious dose
TGBE	tris-glycine beef extract
THMs	trihalomethanes
tLOD	theoretical limit of detection
TSB	tryptone soy broth
TSP	trisodium phosphate
TSS	total suspended solids
TV	Tulane virus
UGent	Ghent University
UK	United Kingdom
UNG	uracil-N-glycosylase
USA, US	United States of America
USDA	US department of agriculture
UV	ultraviolet
VIRADEL	virus adsorption/elution
VLP	virus-like particles
vMC_0	genetically modified mengovirus
VT-	verotoxin negative
VTEC	verotoxin producing *Escherichia coli*
WB	washing bath
WHO	World Health Organization
W_s	Wilcoxon signed-ranks (test)
WWTP	wastewater treatment plant

OBJECTIVES AND OUTLINE

OBJECTIVES AND OUTLINE

THESIS OBJECTIVES

For this thesis funding was received from a PhD grant of the Agency for Innovation by Science and Technology (IWT), the SPF Public Health TRAVIFOOD project, and from the European Community's Seventh Framework Program (FP7) (no 244994) Veg-i-Trade project. The EU FP7 Veg-i-Trade project as a whole project dealt with the impact of climate change and globalization on safety of fresh produce (www.veg-i-trade.org). The research work executed in the present PhD thesis contributed in particular to Work Package 6 of the Veg-i-Trade project on microbial analysis and risk assessment studies, including Norovirus in raspberries and lettuce. These two case studies have also been identified by EFSA as the two most frequent combinations of foodborne pathogen and food of non-animal origin (FoNAO) reported in outbreaks in the EU (2007-2011): N°1: raspberries and NoV (27 outbreaks); N°2: leafy greens eaten raw as salads and NoV (24 outbreaks) (EFSA 2013).

The aims of this PhD project were

(i) To identify the contamination sources introducing NoV in the fresh produce chain, taking into account the persistence of viruses in the environment and food, and the effect of treatments used in the processing of fresh produce.

(ii) To provide new data concerning sampling and testing for NoV in the fresh produce chain.

(iii) To identify the risk for NoV contamination associated with the use of water in the farm-to-fork chain of fresh produce.

The objective was also to provide information that is vital for the identification of effective mitigation strategies. As such while completing the three objectives listed above, this PhD thesis will give a perspective on the relevance of NoV in fresh produce and will provide knowledge on the prerequisites and critical points during the production chain of fresh produce for NoV contamination, persistence, inactivation and removal.

THESIS OUTLINE

A schematic overview of the general structure of this PhD project is available in Figure 0.1. In **Chapter 1** a literature overview is presented to introduce the foodborne pathogen 'norovirus' and to justify the relevance of NoV in foodborne outbreaks (FBO) due to fresh produce. Furthermore transmission routes and viral persistence for NoV during the farm-to-fork chain of fresh produce are reviewed and discussed as a basis to identify possible prevention and control efforts for risk mitigation. In order to grasp the influence of the whole farm-to-fork chain on the viral load of fresh produce, also the effect of treatments used in food processing of fresh produce on viruses was described. The latter shows the relevance of "good practices" to limit viral (cross-)contamination and enables possible identification of intervention strategies post-harvest.

In order to provide new data concerning *sampling and testing for NoV* in the fresh produce chain, first the necessary detection methods were selected and evaluated. Detection methods for NoV in foods, including fresh produce such as soft red fruits and leafy greens, have been extensively studied in prior PhDs at the Dept. of Food Safety and Food Quality at Ghent University (Baert 2009; Stals 2011). As such, the in-house detection methodology for NoV in fresh produce was selected for NoV detection in raspberries and lettuce. This method is based on the elution-concentration method and the use of murine norovirus (MNV-1) as process control and MNV-1 RNA as amplification and inhibition control (Stals et al. 2011). As identified in Chapter 1, both water and surfaces (including hands) can be a source of NoV contamination. Hence in **Chapter 2** a detection strategy for NoV in irrigation and processing water was selected and in **Chapter 3** some detection methods for NoV on surfaces were assessed. The methods selected during this PhD study for fresh produce, water and surfaces have been used in the EU FP7 Veg-i-Trade project during sampling and analysis.

As limited data is available for NoV presence in fresh produce unrelated to outbreaks, this PhD project also contributed to data gathering on the presence of NoV in raspberry products. However in contrast to most available studies a multi-sample approach was undertaken in **Chapter 4** instead of a single sample survey. A batch testing regime (n=5, c=0) (as often applied in import controls or inspections by competent authorities) was undertaken on 26 random batches of frozen raspberries derived from four raspberries' processing companies. Furthermore a decision tree was elaborated to support interpretation of NoV RT-qPCR results.

During the literature study in Chapter 1, both infected food handlers and contaminated water were identified as relevant transmission routes for NoV in the fresh produce chain. In literature ample experimental studies with quantitative data are now available that have simulated the transfer of viral contaminated fingers to food products and surfaces (reviewed in Kotwal and Cannon 2014). Also food handlers have been frequently identified during FBO investigations as the source of contamination. However, in contrast similar quantitative information to model the transfer due to the use of viral contaminated water is much less available in literature and is missing in case of fresh produce processing water. Also viral FBO that are directly linked to the use of contaminated water at primary production or during processing are missing, although in some cases Norovirus contamination of the fresh produce at farm level was suggested to be caused by contaminated water (e.g. in Ethelberg et al. 2010). However, due to the difficulty of source tracking further in the fresh produce chain, actual viral genomic copies by analysis have not yet been identified in the water suspected as viral contamination source. Hence the goal of the second part of this PhD study was to further elucidate the potential role of *water as a transmission route* for viral pathogens.

In **Chapter 5** the role of processing water as a (cross-)contamination pathway during the washing of lettuce was explored. Quantitative data was gathered on the cross-contamination of (surrogate) virus in comparison to bacteria during the washing of lettuce in a simulation of an industrial two-step wash process. A worst-case situation was modeled since no appropriate sanitizers were included in the washing process. However this worst-case situation is relevant since it is still applied in several European countries (e.g. Belgium and the Netherlands).

In order to further grasp the current knowledge on water as a transmission route, a review was performed in **Chapter 6** on the available quantitative microbial risk assessment studies in peer-reviewed literature that included the modeling of effect of water use (e.g. influence of irrigation water and/or washing step) or water treatment on the quality of fresh produce in at least one stage of the farm-to-fork supply chain. This literature overview was the result of the discussion group of the International Life Sciences Institute (ILSI) working group 'Quality of water used in the production of fresh produce' (http://www.ilsi.org/Europe/Pages/Emerging-Microbiological-Issues-Expert-Groups.aspx) and was not limited to the risk implied by the presence of viral pathogens, as also pathogenic bacteria, parasitic protozoa and helminths were included. This to have a better idea on the modeling strategies generally applied in QMRA to model this transfer of pathogens in water to fresh produce. Attention was likewise given to the different

strategies used to model other events in the food chain of fresh produce. Also relevant mitigation strategies, recurring proxy data, assumptions, and data gaps were identified and summarized. During this review many recurring data gaps were noted that still exist, and from previous chapter on detection methods it is clear that it is still debatable how to interpret data on NoV (presence of genomic copies detected by molecular methods) to public health risk. Therefore, it was decided in the framework of this PhD study that the elaboration of a risk assessment study on the use of water in leafy greens production and processing was currently of no added value.

Finally, in **Chapter 7** a general discussion integrating the findings, conclusions and recommendations obtained during this PhD project is presented. Special attention was reserved for discussion on the uncertainties and linked challenges one is confronted with when striving for a holistic understanding of NoV in the farm-to-fork chain of fresh produce. Uncertainties discussed relate to health risk, prevalence & testing, relevance of indicators & control, and standardized detection of NoV.

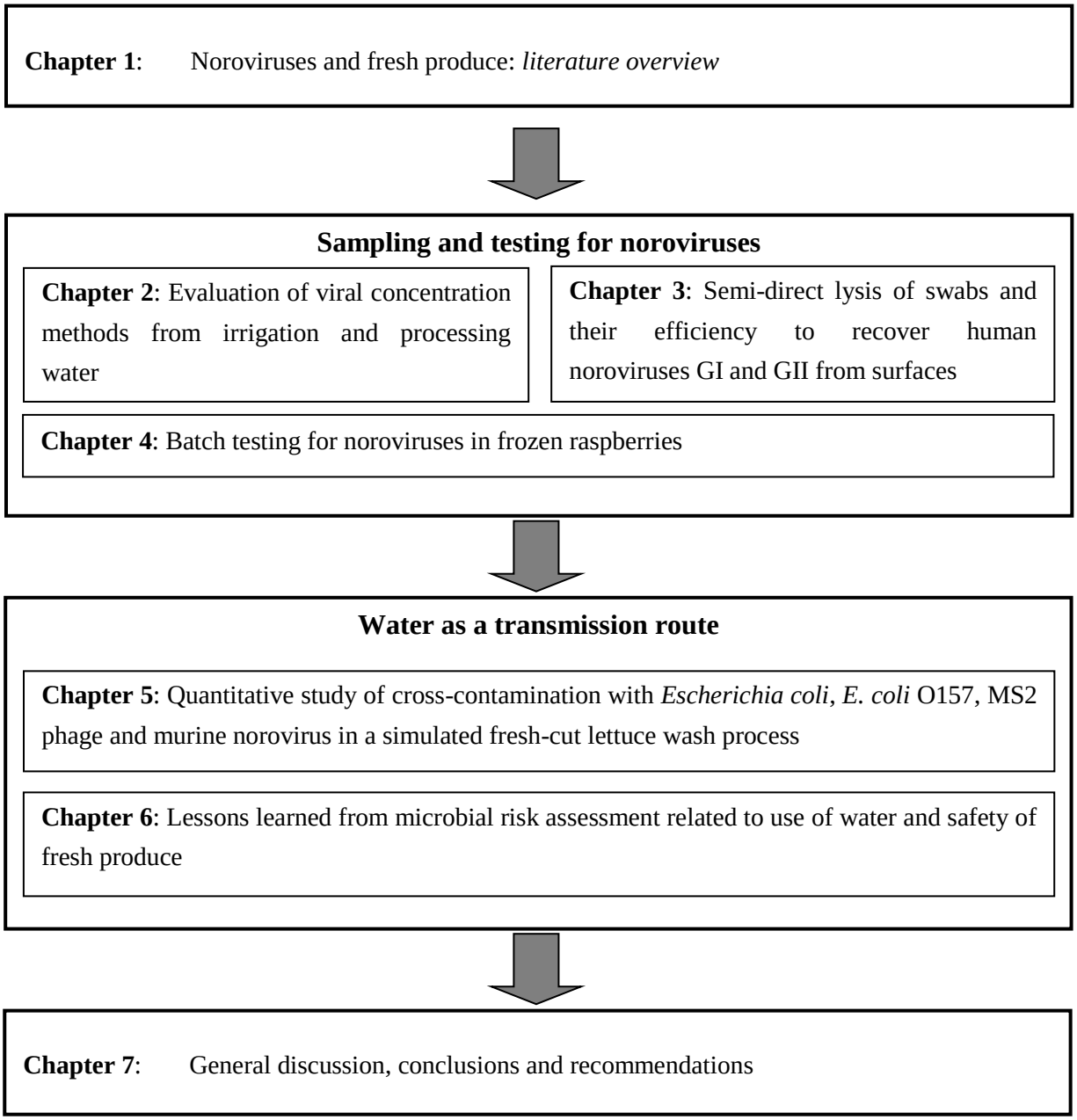


Figure 0.1. Research outline of this PhD project.

CHAPTER 1

NOROVIRUSES AND FRESH PRODUCE:
LITERATURE OVERVIEW

1. NOROVIRUS AND FRESH PRODUCE: LITERATURE OVERVIEW

1.1. EPIDEMIOLOGY AND DETECTION OF FOODBORNE VIRUSES

Foodborne viruses can be defined as human infecting viruses transmitted through food. The viruses implicated in foodborne disease are enteric viruses, which represent a wide spectrum of viral genera that invade and replicate within cells of the intestinal tract, subsequently they attack other cells of the digestive tract or enter other organs such as the liver or central nervous system and cause disease. Infection can result in a wide variety of symptoms ranging from a mildly, self-limiting gastroenteritis, to hepatitis, aseptic meningitis, and acute flaccid paralysis. Overall, these foodborne viruses are excreted in high numbers in human feces, and are transmitted by the fecal-oral route. Viruses are strict intracellular parasites and cannot replicate outside a specific living cell. Therefore the number of viral particles does not increase in food and water during production, processing, transport, and storage. Furthermore, viruses are inert and metabolic non-active when outside their hosts, and as such no sensory changes are noticed when present in food. Foodborne viruses consist of genomic material encapsulated in a protein shell as schematically presented in Figure 1.4. (a). These enteric viruses lack an envelope of lipids, which contributes to their environmental persistence.

Several enteric viruses have been linked with foodborne outbreaks (FBO), including e.g. rotavirus (e.g. Mayr et al. 2009), sapovirus (e.g. Yamashita et al. 2010), astrovirus (e.g. Oishi et al. 1994), poliovirus (e.g. Dhanashekar et al. 2012), enterovirus (e.g. Bellou et al. 2013), aichivirus (e.g. Yamashita et al. 1991), and hepatitis E virus (e.g. Tamada et al. 2004). However the two most frequently linked viruses with FBO, and as such identified as the foodborne viruses with the highest priority are norovirus (NoV) (Figure 1.1.) and hepatitis A virus (HAV) (FAO/WHO 2008b; Koopmans and Duizer 2004). No zoonotic transmission has been observed so far for NoV and HAV (Mathijs et al. 2012), and hence transmission for NoV and HAV is directly by person-to-person or indirectly via human fecal contaminated food, water, or surfaces. In case of NoV, transmission can also occur via vomit and aerosol formation after projectile vomiting, next to the fecal-oral route. Due to their high relative importance as viral foodborne pathogens, both NoV and HAV will be discussed briefly in this subsection 1.1. However the emphasis of this literature overview will be on NoV as this is the subject of this PhD study.

1.1.1. Norovirus & hepatitis A virus

1.1.1.1. NoV, "the Ferrari of the foodborne virus world"

NOV CLASSIFICATION AND CHARACTERISTICS

Human noroviruses (NoV), the main subject of this PhD thesis, belong to the genus *Norovirus*. NoV are non-enveloped, positive-sense, single-stranded RNA viruses with a genome of approximately 7.5 kb composing, with the exception of murine norovirus (4 ORFs), of three open reading frames (ORFs) (Karst et al. 2015). Together with the genera *Sapovirus*, *Lagovirus*, *Vesivirus*, *Nebovirus* (Oliver et al. 2006), the newly proposed *Recovirus* (Farkas et al. 2008), and other tentative new genera (e.g. *Valovirus* (L'Homme et al. 2009)) and unclassified viruses, the *Norovirus* genus forms the *Caliciviridae* family. The genus *Norovirus* consists of six genogroups (GI to GVI), of which three (GI, GII and GIV) are known to cause illness in humans. Next to human infective genotypes, GII and GIV consist also of genotypes infecting respectively swine (GII.11, GII.18, and GII.19) and lions (Martella et al. 2007; Wang et al. 2005). Remark that these genotypes are uniquely related to animals and are hence non-human infecting subtypes within genogroup GII and GIV. Bovine and murine NoV (MNV) are classified respectively in genogroup III (GIII) and V (GV) (Oliver et al. 2003; Karst et al. 2003). Recently, canine noroviruses (CNVs) have been classified into GIV and the tentative new GVI (Mesquita et al. 2010; Martella et al. 2008). Mainly GI and GII genotypes, comprising over 20 different genotypes (Zheng et al. 2006), are responsible for the majority of NoV (foodborne) outbreaks. Among these genotypes the GII.4 variants are responsible for the majority (estimates of 55-85%) of NoV gastroenteritis cases worldwide (Ramani et al. 2014).

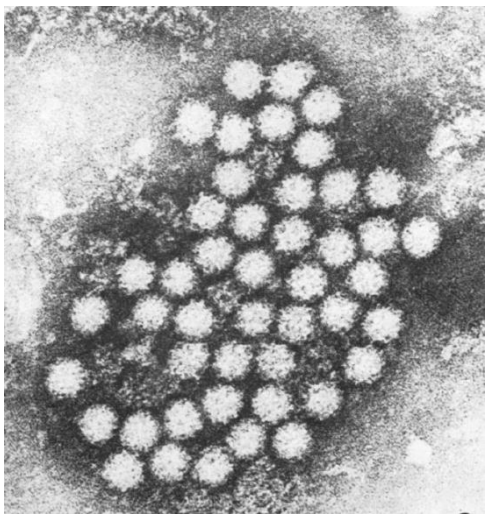


Figure 1.1. Immune electron microscopy picture of NoV (27 – 32 nm). Source: Kapikian et al. 1972

The first glimpse of a member of the now called *Norovirus* genus was spotted in 1972 through an immune electron microscopy in a stool sample filtrate derived from an outbreak in Norwalk Ohio in 1968. Since then, this viral agent of 27 – 32 nm was named after the place where it was discovered resulting in "Norwalk virus", now recognized as the type agent Norwalk virus (NV) belonging to GI.1 (genogroup I, genotype 1) (Kapikian et al. 1972; Kapikian 2000). However, the taxonomic status remained uncertain until the cloning of the NV genome in 1990 (Xi et al. 1990) which lead to the classification of NV and other related "small round structured viruses" (SRSVs) as members of the *Caliciviridae* family (Green et al. 2000). As such, depending on the timeframe, members of the *Norovirus* genus have been commonly referred to as Norwalk viruses, "small, round structured viruses" (SRSV), Caliciviruses and Norwalk-like viruses (NLV). This diversity in naming along the last 40 years can also be observed in outbreak Table 1.1. The observation that recombination of NoV in the ORF1-ORF2 junction region is common (Bull et al. 2007) has recently triggered a new proposal for a unified NoV nomenclature and genotyping to be able to recognize these recombinant viruses. The new proposal is a dual typing system based on complete capsid (VP1) and partial polymerase (1300 nt) sequencing (Kroneman et al. 2013).

Since its discovery and especially since the development of molecular detection methods (e.g. RT-PCR) in the nineties, the notorious star of noroviruses has risen fast among the echelon of foodborne disease agents. Nowadays NoV are known as the "winter vomiting bug", the "cruise ship virus" and have even been termed "the Ferrari of the virus world" (Kelland 2012). This virus is considered the most prevalent cause of viral gastroenteritis and even considered to be the primary cause of overall gastroenteritis (Koopmans and Duizer 2004). Human noroviruses are generally detected in human wastewaters all over the world all year round, indicating epidemiologically a global presence and persistence in the community. This global presence is also highlighted by studies on the seroprevalence to GI and GII strains in e.g. Canada (Cubitt et al. 1998), UK (Menon et al. 2013a), Italy (Pelosi et al. 1999), Finland (Nurminen et al. 2011), India (Menon et al. 2013b; Menon et al. 2013a), and Korea (Son et al. 2013), demonstrating that when reaching adulthood nearly all adults have been exposed to one or more NoV (Donaldson et al. 2010). Remark that serologic tests in these studies are based on ELISA and hence results can depend on the virus-like-particle (VLP) that was selected as antigen. As such, a limitation of the used test is that a specific type of VLP, e.g. of GII.4, is not necessarily able to detect cross-reactive antibodies against non-GII.4 NoV strains, including GI (Mesquita and Nascimento 2014).

NoV have a particular set of characteristics compared to other bacterial and viral pathogens that enable them to spread easily during (foodborne) outbreaks and hence contribute to the global presence of this viral pathogen (Patel et al. 2009). As such, **(i)** NoV have a very low infectious dose facilitating easy transfer of significant doses by simple contacts, e.g. by food handlers. Based on data resulting from a human experimental infection study using NV, the average probability of infection for a single NoV particle was estimated to be close to 0.5, exceeding that reported for any other virus studied up to date (Teunis et al. 2008). A second factor **(ii)** contributing to the success of NoV is due to characteristics of the shedding of viral particles. As such, the viral load found in feces from infected persons is relatively high (up till 10^{12} viruses (RT-PCR) per gram feces (Atmar et al. 2008)) and the shedding can continue for a prolonged duration even after symptoms have resolved. Also asymptomatic infections and shedding are frequently observed, increasing the likelihood of food handlers to be responsible for 'silent' transmission in the case of insufficient respect of good personnel hygiene. Next to feces, NoV viral particles are also shed in vomitus, which introduces the possibility for spread by ill persons via vomit-contaminated surfaces. The dramatic nature of the vomiting episodes (projectile vomiting) typical for a NoV infection, produces also a lot of aerosolized vomit, increasing the risk for widespread contamination and airborne transmission and hence secondary spread. Secondary attack rates as high as >30% are known to occur among contacts of infected persons (Glass et al. 2009). A third characteristic favoring NoV **(iii)** is its prolonged environmental stability. As NoV have the ability to evade death from cleaning and disinfection, i.e. particular resistant to normal household disinfectants and even alcohol hand gels, and persist long periods outside a human host, even at wide temperature ranges (from freezing to 60°C). Also **(iv)** the substantial strain diversity, the lack of complete cross-protection against the diverse strains, the inadequate development of long-term immunity, and antigenic drift enable the virus to escape pre-existing herd immunity or infect previously naive populations (Donaldson et al. 2008; Karst et al. 2015) and contribute to the possibility of repeated infections throughout life following re-exposure with NoV (Patel et al. 2009). The great diversity of strains is attributed both to the accumulation of point mutations associated with error-prone RNA replication due to the lack of proofreading activity of the NoV RNA dependent RNA polymerase, and to recombination between two different NoV strains (Bull et al. 2007; Glass et al. 2009). Antigenic drift has been observed for the predominant GII.4 NoV strain, but may also occur in other genotypes (e.g. GII.2 (Iritani et al. 2008)). As such, from the 1990s till early 2013, seven different GII.4 variants were associated with global epidemic of gastroenteritis (Ramani et al. 2014). Due to this rapid evolution of NoV and the great diversity of strains, in contrast to HAV, no effective vaccine is yet available. NoV virus-like particles (VLPs)

have been tested as a candidate vaccine and have shown efficacy in a proof-of-concept human experimental infection model. However, despite the several advances that have been made in vaccine development in recent years, many challenges remained to be solved (Ramani et al. 2014).

DIAGNOSIS AND PROGNOSIS OF INFECTION AND ILLNESS

Concerning the clinical features of a NoV infection, typically gastroenteritis starting with a sudden onset of vomiting (more common in children) and/or diarrhea (more common in adults) is observed after a median incubation period of 1.2 days (95% CI 1.1-1.2 days) (Lee et al. 2013). Although the predominant symptom can vary from person to person. Nausea, abdominal pain, abdominal cramps, malaise, and low-grade fever also occur. Generally, NoV disease is characterized as mild and self-limiting, and symptoms lasting 2-3 days (Koopmans et al. 2002).

A recent *in vitro* study revealed that the current globally dominant GII.4 NoV strain infects more specific human B cells and that the presence of certain enteric bacteria are likely needed to facilitate productive attachment to, and infection of these B cells (Jones et al. 2014). Infection with NoV can cause malabsorption of water from the gut, hence resulting in diarrhea. However, available data suggest that human NoV-induced diarrhea is not caused by structural damage of the intestinal wall but instead by alterations of secretory and/or absorptive processes (Karst et al. 2015). Diarrheal stools usually contain no blood, mucus, or leukocytes. The absence of leukocytes differentiates NoV associated diarrhea from diarrhea caused by bacterial pathogens such as *Escherichia coli* O157:H7, in which blood appears in the stool (Glass et al. 2009). Feces does contain viral particles up to a median of 9.5×10^{10} genomic copies per gram feces as measured by RT-qPCR during an inoculation study with Norwalk virus (GI.1) (Atmar et al. 2008). Shedding has been observed to start in the pre-symptomatic phase (3-14 hours before onset), and has been shown to last for up to 56 days post-infection (detection by RT-PCR) (Atmar et al. 2008). However, this shedding may even be prolonged in immunocompromised persons. Approximately one-third of human NoV-infected individuals are asymptomatic (Hall et al. 2011; Graham et al. 1994), resulting in a high incidence of asymptomatic infections in the community (1%-16%) (Glass et al. 2009; Phillips et al. 2010). This leads to further propagation of disease as shedding still results in high titers in feces of asymptomatic infections (e.g. 10^6-10^8 copies/g of stool (Ozawa et al. 2007)). As such it is virtually impossible to exclude viral shedders solely on the basis of visible symptoms.

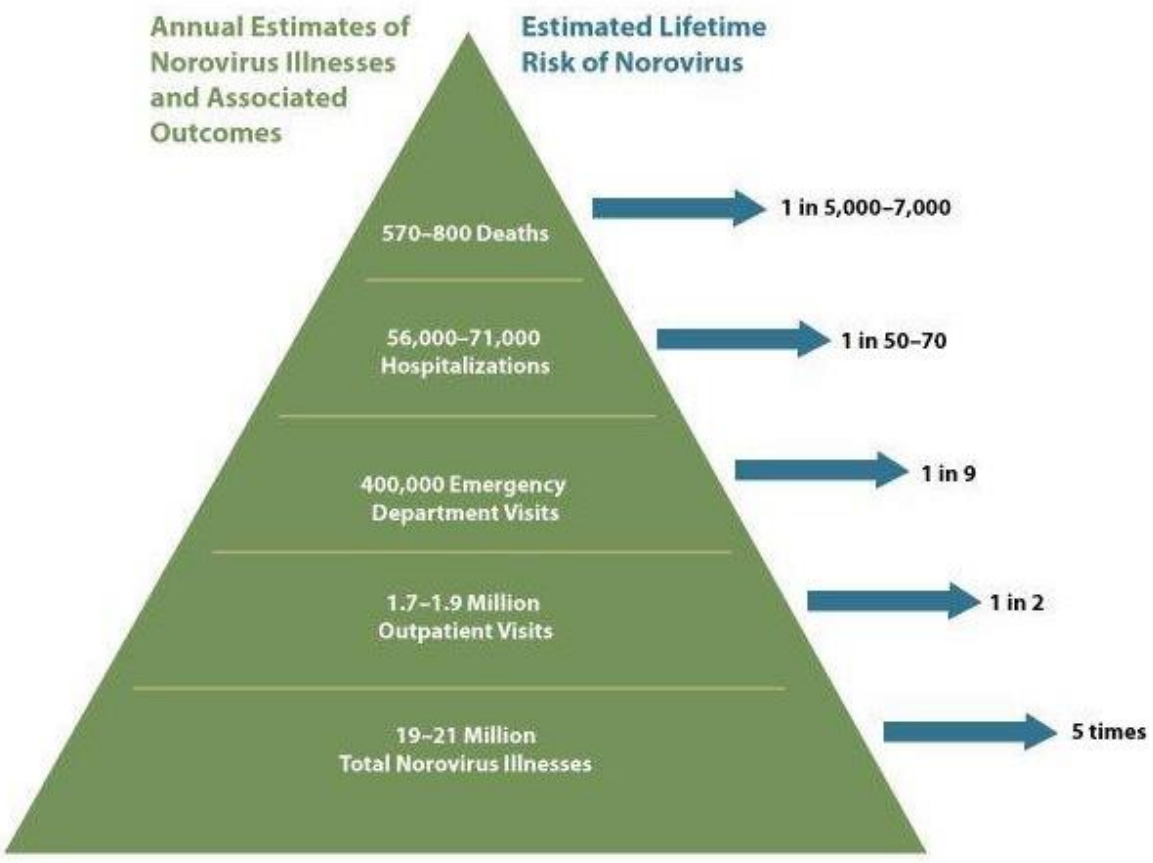


Figure 1.2. Burden of NoV in the United States. Estimates of the annual number of illnesses and associated outcomes for NoV disease in the USA, across all age groups. Lifetime risks of disease are based on a life expectancy of 79 years of age. Source: CDC 2014.

Although NoV infection is regarded as a mild and self-limiting disease, in some cases supportive therapy to prevent dehydration (e.g. parenteral fluid therapy) or even hospitalization is required. Deaths are rarely associated with foodborne NoV outbreaks (death rate is 0.01% in USA) (Hall et al. 2014) and are more commonly associated with person to person outbreaks among elderly and in health care facilities (Trivedi et al. 2013). In the EU in 2012 a total of the 101 strong-evidence NoV FBO were reported by 20 of the 27 reporting member states and two non-member states (i.e. Norway and Iceland), resulting in 13 853 human cases, 122 hospitalizations and 2 deaths (EFSA and ECDC 2014).

Next to naturally acquired immunity (although observed to be short-lived), susceptibility to the majority of NoV infections is determined by genetically controlled secretor-dependent expression of histo-blood group antigens (HBGAs) (Shanker et al. 2014). HBGA have been considered as an important co-factor necessary for NoV attachment to host cells (Donaldson et al. 2008) and are localized on the surface of epithelial cells and in mucosal secretions of secretor-positive individuals. Specific HBGA expression on cell surfaces is affected by the ABO, Secretor and Lewis genotypes of an individual. Non-secretor

individuals are believed to be largely resistant to infection with many human NoV genotypes (Ramani et al. 2014). However a recent study suggests that HBGA-expressing *bacteria* may play a vital role as cofactor to facilitate NoV infection of B cells (Jones et al. 2014). Anyway cases of NoV infection associated with secretor-negative individuals are reported (Jin et al. 2013; Nordgren et al. 2010, 2013). It has also been suggested that, due to the variation between norovirus genotypes, it is likely that every person is genetically susceptible to at least one NoV genotype (Atmar 2010).

DIAGNOSIS OF AN OUTBREAK

Primary attack rates in foodborne outbreaks are generally high (typically ca. 50%) (Koopmans et al. 2002; Matthews et al. 2012). For diagnosis in the absence of extensive laboratory testing for detection of NoV, careful evaluation of clinical and epidemiological characteristics of outbreaks can allow rapid identification of causative agents and thus helps to take the precautionary steps needed and to guide the public health laboratory to conduct appropriate tests to confirm the presence of NoV (Hedberg 2006). Kaplan described a list of epidemiological and clinical criteria that proved to be indicative of norovirus outbreaks: (i) stool cultures are negative for bacterial pathogens; (ii) median incubation period 24-48 h; (iii) median duration of illness 12-60 h; (iv) vomiting in >50% of patients (Kaplan et al. 1982). These criteria can be used for rapid prospective and retrospective evaluation of outbreaks and have been observed to be highly specific (98.6%) and to have a high positive predictive value (97.1%) (Turcios et al. 2006).

Nowadays confirmation of NoV foodborne-disease outbreaks, according to the guidelines of the CDC, requests the detection of viral RNA in at least two bulk stool or vomitus specimens by real-time or conventional reverse transcriptase-polymerase chain reaction (RT-PCR), or visualization of the viruses with characteristic morphology by electron microscopy in at least two or more bulk stool or vomitus specimens, or two or more stools positive by commercial enzyme immunoassays (CDC 2000).

RT-PCR assays are used when sequencing is wanted for genotyping and further molecular epidemiologic studies. This is helpful for epidemiologic studies and especially to detect international outbreaks as strain sequences from outbreaks linked to a common source are expected to be more similar than strains from outbreaks with a different source (Verhoef et al. 2011). When using commonly applied RT-qPCR assays (ISO/TS 15216-1:2012), this allows a more rapid evaluation of clinical samples, but classifies NoV to the genogroup level (Atmar 2010).

Next to commercial molecular assays for RT-(q)PCR (e.g. Norovirus Screening RT-PCR assay (CeeramTools®, France); RealStar® Norovirus RT-PCR Kit 1.0 (Altona Diagnostics

GmbH, Germany); Xpert® Norovirus (Cepheid)), several commercial immunoassays are available on the market (e.g. RIDA®SCREEN (R-Biopharm), RIDA®QUICK (R-Biopharm), IDEIA assays (Oxoid)). Although these enzyme immunoassays (EIA) are less sensitive, they can be used as screening assays during outbreak situations as normally multiple feces samples are tested which improves the likelihood of obtaining a positive result (Duizer et al. 2007). However, it is suggested that these results should be confirmed by RT-PCR (Gray et al. 2007; Hall et al. 2011).

1.1.1.2. Hepatitis A virus

HAV CLASSIFICATION AND CHARACTERISTICS

HAV is a viral agent consisting of a non-enveloped capsid of 27-32 nm in diameter containing a positive single-stranded RNA genomic molecule of 7.5 kb, composing of a single open reading frame. HAV is the only member of the *Hepatoviruses*, which is a genus within the Picornaviridae family. Other genera within this family include *Rhinovirus* and *Enteroviruses*. HAV isolates are characterized by a low antigenic variability, resulting into the recognition of only a single serotype. However several genotypes have been identified. Recently HAV have been reclassified in six genotypes based on the sequence derived from the complete VP1 region. Genotypes I-III have been associated with infections in humans, while genotypes IV-VI are simian in origin. Genotypes I, II and III can each be further divided in subtypes A and B. The predominant subtype is dependent on the geographical region, however subtype IA seems to be responsible for the majority of hepatitis A cases worldwide (Vaughan et al. 2014).

Like NoV, HAV is known for its environmental persistence, a feature that facilitates spread by means of contaminated food products and water. The infectious dose is estimated to be in the range of 10 – 100 viral particles (Sanchez et al. 2007).

DIAGNOSIS AND PROGNOSIS OF INFECTION AND ILLNESS

Hepatitis A is a self-limiting disease that results in fulminant hepatitis, however deaths are associated with HAV infection. As such, the fatality rate of foodborne HAV infection is estimated to be 2.4% in the USA (Scallan et al. 2011). Also a higher hospitalization rate (i.e. 31.5%) is notable compared to NoV infections (i.e. 0.03%) (Scallan et al. 2011). Clinical symptoms are dark urine, fatigue, anorexia, abdominal discomfort, and nausea, followed by symptoms and signs of hepatitis 1-2 weeks later (i.e. jaundice). However infection with the same virus leads to variable disease courses in different individuals. Illness can last from a few weeks to several months (e.g. up to 6 months in 10-15% of patients) and can lead, however infrequently, up to fulminant hepatitis (Koopmans et al. 2002). Yet in children under six the infection is generally asymptomatic. As such, the burden to human health related to a HAV infection is generally much higher in comparison

with a mild acute gastroenteritis (AGE) related to NoV infections (Havelaar et al. 2012). Compared to NoV infections, HAV has also a long incubation period of 2–7 weeks, complicating epidemiological investigations to link outbreaks with a specific food item (Koopmans et al. 2002; Sanchez et al. 2007). In large parts of the world HAV is endemic (WHO 2014), primarily in developing countries with poor sanitary and hygienic conditions, and as a result virtual all adults are immune (immunity is life-long, only one serotype of HAV) due to contact during early childhood.

The primary site of replication of HAV is the liver, which they reach following a viremic stage in which the virus can be detected in the blood stream, and where they infect hepatocytes. After replication in the liver, the virus is released into bile and, eventually, shed in feces (Koopmans et al. 2002; Vaughan et al. 2014). As such feces can contain high numbers of virus particles (10^6-10^8 particles/g feces) during infection. Pre-symptomatic shedding can start in the last two weeks of the incubation period and can proceed up to five months after infection. But next to the fecal-oral transmission route, occasionally HAV is also acquired through blood transfusions or sexual contact (anal-oral) (Sanchez 2013).

In contrast to NoV, effective vaccinations providing long-lasting immunity against HAV are available on the market since early 1990s. However these are not overall administered on a national level due to varying cost-benefit calculations (Poovorawan et al. 2002). As these vaccines, consisting of inactivated viruses, have quite high economic costs and hence highly endemic countries that usually have low economic incomes cannot afford the hepatitis A vaccination (Sanchez et al. 2007). Nevertheless vaccination should be considered for special groups of persons that are at a high risk of HAV infection (e.g. travelers, researchers, chronic liver diseased persons). Vaccination of food handlers has been proposed as a mitigation strategy for FBO (Poovorawan et al. 2002). However, such policies have been suggested not to be cost-effective in developed countries (Meltzer et al. 2001).

DIAGNOSIS OF AN OUTBREAK

Diagnostic confirmation during foodborne-disease outbreaks is typically done by detection of immunoglobulin M antibody to HAV (IgM anti-HAV) in serum for two or more persons who are presumed to be linked to the implicated food (CDC 2000). However molecular detection methods are also needed to detect the viral pathogen in food (ISO/TS 15216-1:2012) and for HAV sequencing in human and food samples. The latter can facilitate in identifying nationwide outbreaks as was the case in the recent multi-member state outbreak in Europe identified by HAV sub-genotype IA with an identical RNA sequence (sequence GenBank number is KF182323) (ECDC 2014). However, in order to facilitate future nationwide comparison of HAV sequences, the need for the development of a harmonized

analytical protocol for HAV sequencing in human and food isolates was identified as a point for improvement (ECDC 2014).

1.1.2. Epidemiological evidence linked to fresh produce

NoV are responsible for 47-96% of outbreaks of acute gastroenteritis reported in countries around the world (Atmar and Estes 2006). However only a fraction is due to foodborne transmission, e.g. approx. 22% and 23% of NoV outbreaks in Europe and the USA respectively (Hall et al. 2014; Verhoef et al. 2009). However in a recent study by UK FSA only 2.7% of NoV outbreaks in the UK was estimated to be foodborne (Tam et al. 2014). Concerning HAV outbreaks, these estimates concerning a foodborne transmission are generally lower, i.e. 7% of domestically acquired illnesses in the USA (Scallan et al. 2011) and as high as 16% of reported case-patients in the Netherlands (Petrignani et al. 2014) when additional virus typing was implemented. The number of foodborne outbreaks (FBO) due to viruses is likely underestimated since secondary transmission can mask the connection between sources and outbreaks, resulting that outbreaks initially linked to a food-source may present as person-to-person outbreak by the time they are recognized (Verhoef 2011). A FBO is hereby defined as an incident in which two or more persons experience a similar illness resulting from the ingestion of a common food (CDC 2000).

During 2009-2012, NoV were deemed responsible for 48% of FBO with a single suspected or confirmed cause reported in the USA (Hall et al. 2014). In 2012, NoV completed the top three of most frequently reported causes of FBO in the EU, after *Salmonella* and bacterial toxins (EFSA and ECDC 2014). However, NoV outbreaks were responsible for the highest number of human cases (56.7% of reported cases), mainly due to the largest FBO that year, affecting 10950 people due to NoV contaminated frozen strawberries (Bernard et al. 2014; EFSA and ECDC 2014). Also in the USA NoV are responsible for the majority (estimate of 58%) of all domestically acquired foodborne illnesses attributed to a known agent (Scallan et al. 2011).

Fresh produce has also, next to shellfish and ready-to-eat foods, been identified as an important vehicle for the foodborne transmission of enteric viruses such as NoV and HAV (Bassett and McClure 2008; FAO/WHO 2008b). As such, vegetable row crops (e.g. leafy greens), fruits and mollusks, were responsible for 30%, 21%, and 19%, respectively of NoV FBO in the USA (2009-2012) in which a specific food category was implicated (Hall et al. 2014). Concerning fresh produce outbreaks, NoV was identified as the top cause of outbreaks (40%), followed by *Salmonella* (18%) and *E. coli* (8%) according to a comprehensive survey of outbreaks with identified food sources in the USA (1990-2005) (Dewaal and Bhuiya 2009).

In this section further epidemiologic evidence based on the outbreak Table 1.1. consisting of peer-reviewed outbreak investigation studies, will be presented to highlight the relevance of NoV and HAV as pathogenic agents in FBO due to contaminated fresh produce.

BIAS

This overview of peer-reviewed outbreak investigation literature gives only a narrowed view on the relevance of fresh produce as a vehicle for viral FBO. As these studies only represent a fraction of the fresh produce outbreaks attributed to NoV and HAV. As such, in Europe (2007-2011) at least 27 FBO were linked to NoV contaminated raspberries (EFSA 2013), while only two outbreaks fitting this geographic element and timeframe are mentioned in Table 1.1. Likewise the variety of fresh produce types linked to viral outbreaks is evidently higher than depicted in Table 1.1. as other associated fresh produce commodities include e.g. cantaloupe (Bowen et al. 2006), tomatoes and carrots (EFSA 2013; Erickson 2010). Hence not every FBO is reported in peer reviewed literature and it has been observed that an outbreak has a higher chance of publication if certain pre-requisites are fulfilled such as: (i) high number of patients affected, long-lasting epidemic, or severe illness; (ii) occurrence of unusual types (e.g. genotypes) of the pathogen; (iii) new or unusual food matrices; (iv) the availability of ample analytical evidence or possibility to source-track the origin of contamination. But most of all it sticks out that all reported outbreaks are from North-America or Europe with the exception of reporting countries Israel, Korea and Japan. Hence illustrating the variable capacity and priority to detect viral FBO in the world. Concerning severity of the outbreak, reported FBO due to HAV have likely an edge over NoV outbreaks due to the possible severe nature of the associated illness resulting in a higher hospitalization rate (31.5% versus 0.03%) and a higher death rate (2.4% versus <0.1%) (Scallan et al. 2011). Nevertheless, NoV has been identified to be the top cause of outbreaks (40%) associated with fresh produce in the USA, while HAV contributed to only 4% of produce outbreaks during the same period (1990-2005) (Dewaal and Bhuiya 2009). Hence, this outbreak table is less representative of the real situation as the ratio NoV outbreaks/HAV outbreaks is 28/14. Hence NoV was justly selected as the relevant viral pathogen concerning fresh produce in this PhD thesis.

Even though no genogroup is specified in the report, most probable all outbreaks listed in Table 1.1 that are classified to have NoV as etiological agent will be due to GI or GII NoV. This because most commonly used detection methods, including the ISO/TS 15216-1:2012, only focus on these two genogroups and GIV has to the best of my knowledge not yet been linked to a FBO. The overall prevalence of GIV NoV in the community is unknown, reflecting the lack of including this specific genogroup in general monitoring

programs. However, GIV noroviruses have been found in wastewaters all over the world (e.g. Italy (La Rosa et al. 2010), Japan (Kitajima et al. 2011), France (Sima et al. 2011), Korea (Han et al. 2014)), in stool specimens collected from hospitalized patients with clinical signs of diarrhea (e.g. Italy (Muscillo et al. 2013)), in stool specimens related to outbreaks of AGE (acute gastroenteritis) (e.g. Argentina (Gomes et al. 2007), Australia (Eden et al. 2012)), and in shellfish (e.g. Italy (La Rosa et al. 2012)). This implies the need for further studies to elucidate the role of this virus as a gastroenteritis-causing pathogen and the dynamics of circulation in human populations and environmental contamination.

RELEVANT IDENTIFIED FOOD MATRICES

Frequently identified fresh produce items that were implicated in these outbreaks listed in Table 1.1. are soft red fruits (e.g. raspberries and strawberries) and leafy greens (e.g. salads). Both commodities are also frequently mentioned in European RASFF reports. In the USA, leafy vegetables were identified as the main identified single commodity involved in NoV outbreaks, being responsible for 30% of outbreaks attributed to a single commodity in 2009-2012. Meanwhile fruits were responsible for 21% of single commodity NoV outbreaks (Hall et al. 2014). Hence justifying the selection of soft red fruits (case study: raspberries) and leafy greens (case study: lettuce) as main subjects for this thesis on NoV in fresh produce. The relevance of these two matrices as vehicle of NoV outbreaks is also strengthened by the recent publication of two scientific opinions of EFSA concerning NoV in leafy greens eaten raw in salads (EFSA BIOHAZ Panel 2014b) and NoV in berries (EFSA BIOHAZ Panel 2014a).

Table 1.1. Foodborne outbreak table summarizing outbreaks due to contaminated fresh produce due to NoV or HAV.

Implicated food	Virus	Location	Year	Origin of raw material	Cases (n)/ attack rates (%)	Contamination	References
Lettuce	**HAV**	USA	1988	USA	202 (2 deaths)	Contamination at farm-level	Rosenblum et al. 1990
Salad food	**HAV**	Finland	1996	(imported)	18 & 12 (2 unrelated FBO)		Pebody et al. 1998
Rocket salad	**HAV**	Sweden	2000-2001	(imported)	≥16	Contamination before sale	Nygard et al. 2001
Green salad	**Norwalk virus**	USA	1979		38 (92.7%) and 25 (80.6%) during 2 lunches	Suspected cross-contamination by raw seafood	Griffin et al. 1982
Lettuce	**Norwalk virus**	USA	1981		92	Food handler (FH) suspected	Alexander et al. 1986
Salad	**Calicivirus** genogroup 2	Canada	1998		48 (68%)	Pre-symptomatic FH	Gaulin et al. 1999a
Salad	**NoV**	Israel	1999		159	Infected FH suspected	Grotto et al. 2004
Salads	**Norwalk-like virus** genogroup II	USA (multiple states)	2000		333/ 44%	FH involvement was suggested as cause	Anderson et al. 2001
Salad	**NoV** (GII.7)	Austria	2006		182/ 56%	FH involvement confirmed	Schmid et al. 2007
Mixed salad	**NoV** (GII.4)	UK	2007		34/ 86-95%	FH was ruled out, presence of different subtypes in stool suspected contamination through exposure to sewage.	Showell et al. 2007
Salads	**NoV** (GII.6)	UK	2007		79 (57% - 73%)	FH involvement confirmed (in pre-symptomatic phase during salad preparation)	Vivancos et al. 2009
Salad buffet vegetables	**NoV** (GI.3)	Sweden	2007		413/ 24%	FH involvement confirmed	Zomer et al. 2010
Salad vegetables*	**NoV** (GII.4)	Japan	2007	/	23		Oogane et al. 2008

	Lettuce salad and soup	**NoV** (GII.4)	Portugal	2008		16/ 73%		Mesquita and Nascimento 2009
	Salad	**NoV** (GII.4)	Germany	2009		27	FH involvement confirmed	Wadl et al. 2010
	Lettuce*	**NoV** (GI & GII)	Denmark	2010	France	260/ 54% (11 outbreaks)	All FBO linked to the same kind of lettuce and the same supplier (2 batches) suggesting contamination at **farm-level**. Lettuce was believed to be contaminated with multiple non-zoonotic pathogens (≠ GI & GII NoV, ETEC) leading to the speculation that human fecal matter may have been the source of contamination, possibly via contaminated **water**.	Ethelberg et al. 2010
	Green onions	**HAV** (IA)	USA	1998	Mexico or USA	43	Suggested contamination at farm-level (remark that green onions require extensive handling during harvesting)	Dentinger et al. 2001
	Green onions	**HAV**	USA	2003	Mexico	601/ 18% (3 deaths)	Prior to restaurant delivery; Suspected contamination at farm-level	Wheeler et al. 2005
	Celery component in chicken salad	**Norwalk virus**	USA	/		>1000	Celery component was exposed to non-potable **water**, expected to be the source	Warner et al. 1991
	Potato salad	**SRSV** genotype II	U.K.	/		55 (50%)	Contaminated by contaminated sink used to clean vegetables	Patterson et al. 1997
	Pumpkin salad	**Norwalk-like virus**	Sweden	1999		(27%)	Infected FH	Gotz et al. 2002
	Mixed raw vegetables	**NoV** (GII.1)	Finland	2006	/	> 400 (10 canteens)	Epidemiologic evidence suggests vegetables originating from a single provider as vehicle suggesting contamination before arrival in the canteens.	Makary et al. 2009
	Dried radish salad	**NoV** (GII.4)	Korea	2008		117	Infected FH suspected	Yu et al. 2010
	Cabbage kimchi*	**NoV** (GI.3)	Korea	2011	Korea	451	Ground**water** used for processing of the cabbage was identified as source since GI.3 NoV was detected in the water (homology >99.4% with clinical sample and isolate from kimchi)	Cho et al. 2014

	Frozen raspberries	**HAV**	Scotland	1983	Scotland	24	Suggested contamination at picking stage; cases were reported in the area at the time of picking	Reid and Robinson 1987
	Frozen raspberries	**HAV**	Scotland	1988	Scotland	5/ 71%	Infection confirmed of a picker (FH) at the farm.	Ramsay and Upton 1989
	Raspberries*	**Calicivirus**	Canada	1997	Bosnia	>200		Gaulin et al. 1999b
	Frozen raspberries	**Calicivirus** genogroup 2	Finland	1998	East Europe	509/ 65%	Served as a dressing	Pönkä et al. 1999a; Pönkä et al. 1999b
	Frozen raspberries*	**NoV**	Sweden	2001	/	30	/	Le Guyader et al. 2004
	Frozen raspberries	**NoV** (GI.5)	France	2005		75	Suggested contamination before caterer	Cotterelle et al. 2005
	Frozen raspberries	**NoV** (GII.4, GII.b, GII.7)	Denmark	2005	Poland	1043/ 82% in one of the FBOs (6 outbreaks)	5 of 6 point source FBO linked to the same batch of frozen raspberries from several small farms in Poland	Falkenhorst et al. 2005; Korsager et al. 2005
	Frozen raspberries	**NoV**	Sweden	2006	China	43/ 40%-91% (4 outbreaks)		Hjertqvist et al. 2006
	Frozen raspberries*	**NoV** (GI.4)	Finland	2009	Poland	Ca. 200 (clusters including 3 FBO)	All outbreaks were traced to the same batch of imported raspberries	Maunula et al. 2009
	Frozen raspberries*	**NoV** (GII.4, GII.b, GII.7, GI.4) GII in 12/13; GI in 1/13	Finland	2009	Poland	900/ 49% in one of the FBOs (13 outbreaks)	Some FBO were traced back to the same contaminated batch of frozen raspberries.	Sarvikivi et al. 2012
	Frozen mixed berries (contained suspected pomegranate seeds)	**HAV** (IB)	USA (multistate FBO))	2013	Pome-granate seeds from Turkey	162 (0 death)		CDC 2013
	Frozen mixed berries* in	**HAV** (IA) (sequence	Italy, Ireland,	2013-2014		1444 (23% confirmed):	/	Chiapponi et al. 2014; ECDC 2014; EFSA

	Italy (NL: fresh berries) (FR: mixed berry cake) (Norway: frozen berry mix cake*)	KF182323)	the Netherlands, Norway, France, Germany, Sweden, U.K., Finland			Italy => 90% of cases		2014; Guzman-Herrador et al. 2014 ; Rizzo et al. 2013
	Frozen pomegranate seeds* in raw frozen fruit mix	**HAV** (IB) (sequence KF947077)	Canada	2012	Egypt	6		Swinkels et al. 2014
	Frozen strawberries	**HAV**	USA	1990	USA	28/ 8-10% (2 outbreaks)	Contamination occurred likely by an infected picker (FH)	Niu et al. 1992
	Frozen strawberries	**HAV**	USA (multiple states)	1997	Mexico	242/ 0.2-14%	Possible contamination during harvesting (limited hand hygiene, removed berry calyx with fingernails)	Hutin et al. 1999
	Frozen strawberries	**HAV** (IB) (sequence KC876797)	Denmark, Finland, Norway, Sweden	2012-2013	(strain ≈ sick travelers from Egypt)	103	/	Nordic outbreak investigation 2013; Gillesber Lassen et al. 2013
	Frozen strawberries*	**NoV** (GII.16/II.13 †; GI.9; GII.6)	Germany	2012	China	10 950		Mäde et al. 2013; Bernard et al. 2014
	Raw blueberries*	**HAV**	New Zealand	2002	New Zealand	43	Suggested contamination at farm-level by infected FH or by fecally polluted ground**water** (pit latrines were located in and near orchard, with no hand-washing facilities)	Calder et al. 2003

NoV: norovirus; HAV: hepatitis A virus; SRSV: small round structured virus; ETEC: enterotoxigenic *E. coli* *: the viral agent was also recovered from the food samples; /: data not reported; FH: food handler; †: a recombinant genotype with combination of genotypes II.16 (viral polymerase) and II.13 (viral capsid).

NoV detection in implicated food

Development of detection methods for viruses in food matrices besides shellfish only started in the early 2000s, explaining the lack of viral detection in food matrices linked to FBO in the early years. However the fact that NoV have a low infectious dose, combined with the inability of current detection methods to detect low doses in food, and the possible non-homogeneous contamination within a lot, ensures that even today it is still difficult to detect the viral pathogen in food (both in outbreak and non-outbreak situations). This can also be noted in outbreak Table 1.1. as in only eleven outbreaks the viral pathogen was detected in the implicated food type. The need for further development of sensitive NoV detection methods, combined with the complexity of the food matrix, also hinders sequencing attempts (e.g. in Swinkels et al. 2014). As such virus strain matching in food and patients is rarely obtained for foods other than shellfish (Verhoef 2011), since the level of viral contamination is often too low to allow sequencing. Due to these inherent difficulties, source attribution in most outbreak investigations is mainly based upon epidemiological investigations such as case-control studies.

In 11/42 of outbreaks listed in Table 1.1. food handlers at the catering/large-kitchen stage were suspected or implicated as source of contamination, especially in case of salads and leafy greens. Rarely source tracking was able to pin-point possible contamination routes at farm-level. Although, this route is likely underrated in NoV outbreaks due to inherent difficulties of source tracking and detection of viruses in environmental samples other than stool samples of e.g. food handlers.

Consequences of repeated association of a defined fresh produce commodity with large viral outbreaks

The fact that specific food commodities have been repeatedly linked to large viral outbreaks has had a repercussion on which fresh produce items will be scrutinized more elaborately at the level of import controls performed by government/industry. The 2012 NoV outbreak in Germany involving over 10,000 cases due to the consumption of desserts based upon contaminated frozen strawberries had a repercussion on legal requirements for import control in Europe. As such, from 1 January 2013, a European Union (EU) regulation (Regulation (EC) No 1235/2012) requires 5% of consignments of (frozen) strawberries imported from China into the EU to be tested for NoV and HAV (Anonymous 2012a). Also following the recent multi-member state outbreak of HAV in Europe, possibly linked to frozen berries, implementation of enhanced sampling schemes for frozen berries at the processing and distribution level was considered as a possible mitigation strategy (ECDC 2014).

In the event of an outbreak the action plan can consist of a recall when the implicated food is identified (CDC 2013), but also recommendations for handling frozen soft red fruits have been communicated. In some cases these recommendations are primarily directed to the caterers and large scale kitchens (e.g. in BfR 2013), while in other cases also the consumer is addressed. As such, due to the large outbreaks in four Nordic countries implicating frozen berries, authorities have recommended to boil all frozen berries before consumption as a mitigation strategy (Gillesber Lassen et al. 2013) since the specific implicated batch of berries or berry mix was not yet identified. In Denmark for example, this resulted in an amendment of the decree on food hygiene (Anonymous 2012b). Following the recent multi-member state outbreak of HAV in Europe, recommendation to heat frozen berries before consumption has also been communicated in some of the affected Member States (i.e. Ireland, Italy, Norway) (ECDC 2014; Guzman-Herrador et al. 2014). However, the recommendation for all catering and other large-scale kitchens not to serve unheated frozen berries is not new and has been mentioned earlier in e.g. Pönkä et al. (1999a) and Koopmans et al. (2002). Heating of frozen berries should especially be considered when serving sensitive groups such as in health care facilities.

The increased association of viral outbreaks with soft red fruits in the past years has led to an increased focus on these food commodities and possibly explains partly the high amount of RASFFs reports on soft red fruits, especially in the Nordic countries that experienced multiple outbreaks the past years due to soft red fruits. As such RASFFs do not give an unbiased view on the identity of most commonly associated fresh produce items with viral presence. When searching the RASFFs database for the last 5 years (i.e. January 2010- half September 2014) with search term ‘Norovirus’ as subject, ‘fruits and vegetables’ as product category and ‘pathogenic micro-organisms’ as hazard category, this resulted in 21 reports due to soft red fruits, 2 reports due to leafy greens and no reports due to other fresh produce matrices.

1.1.3. Detection strategy

Investigation of FBO, which includes source tracking, is important to limit current outbreaks and to understand the risk factors present that facilitated the occurrence of this outbreak. A better understanding of how the product became contaminated is crucial for developing long-term prevention measures along the chain. The availability of detection methods for foodborne viruses in the food matrix and in the environment is hence indispensable to facilitate FBO investigations. As such in the German outbreak due to NoV contaminated strawberries in 2012, a timely identification of the implicated food product actively contributed to limit the ongoing outbreak as over 11 tons of strawberries of the incriminated batch could be recalled before it could reach the consumer (Bernard et al. 2014). The availability of detection methods is also important to allow risk-based sampling at processing level as part of the HACCP for viral hazards by e.g. facilitating risk-based sampling of suspected lots of frozen berries originating from affected countries (e.g. endemic countries) and to validate the effect of possible mitigation strategies further in the chain such as e.g. effect of washing on the viral load.

The general strategy for the detection of foodborne viruses in food and environmental samples consists of two steps: (i) virus extraction and/or concentration, and (ii) the detection step.

Remark that during this detection protocol several controls, such as a sample process control virus (PC) and an amplification control, are included as quality control for the efficiency of the extraction/concentration protocol and the functioning of the PCR step (i.e. to control for potential presence of inhibitors of the PCR reaction). The inclusion of these controls together with the necessary negative and positive controls is imperative and as such requested by ISO/TS official method for assurance of the absence of false-negative and false-positive test results. More information on these controls and other controls generally included during the detection step and their interpretation is available in literature (D'Agostino et al. 2011; Anonymous 2012c; Stals et al. 2012a, 2012b). Commonly used viruses as process extraction control include murine norovirus (MNV) (e.g. in Stals et al. 2011a), genetically modified mengovirus (vMC_0) (e.g. in El-Senousy et al. 2013), feline calicivirus (FCV) (e.g. in Mattison et al. 2010) and MS2 phages (e.g. in Scherer et al. 2010). Ideally PC viruses should be: (i) unlikely to naturally contaminate the tested food sample, (ii) safe to handle and easy to cultivate, and (iii) genetically related to the tested virus and ideally have a similar extraction efficiency as the virus under study (Stals et al. 2012a). Other less commonly used sample process control viruses are e.g. bovine enterovirus type 1 (e.g. in Loutreul et al. 2014), PP7 bacteriophage (e.g. in Brandao et al. 2014), canine calicivirus (CaCV), and poliovirus (PV).

In the following two paragraphs a short introduction will be given to the commonly used viral extraction methods in food matrices and in environmental matrices (i.e. surfaces and hands, and water matrix), and the commonly used detection methods. In a last paragraph available data concerning the presence of foodborne viruses in fresh produce (soft red fruits & leafy greens) will be presented. For further information references will be given to relevant reviews in literature.

1.1.3.1. Virus extraction from food and environmental samples

VIRUS EXTRACTION FROM FRESH PRODUCE

For virus detection in food a whole range of virus extraction methods are available. This variety of protocols can be grouped into three main approaches: (i) (acid adsorption) elution – concentration; (ii) direct extraction of the viral RNA from the food matrix, which excludes the elution – concentration step, and (iii) extraction of viruses from the food via proteinase K treatment. For more information on each of these protocols, references are available of relevant reviews (Bosch et al. 2011; Croci et al. 2008; Hartmann and Halden 2012; Stals et al. 2012a). The choice of the ideal extraction method depends on the food matrix under study. For water-based foods such as **fresh produce**, the most popular extraction method is the elution-concentration method, which is also selected in the recent ISO/TS protocol for extraction of HAV and NoV from soft fruits and salad vegetables (ISO/TS 15216-1 (2012)). As the name suggests, the method consist of an elution stage followed by a concentration stage. During elution the food product is brought into contact with an alkaline buffer, which allows the viral particles to detach from the food matrix. When fresh produce is analyzed, frequently pectinase is added to the elution buffer to prevent jelly formation in the eluate by breaking the pectin bonds in fruits and vegetable matrices (Dubois et al. 2002). As the elution process is facilitated only by mild shaking, mainly foodborne viruses on the surface of the fresh produce are targeted. However in situations in which internalization of the viral pathogens is expected other methods should be used that e.g. include homogenization of the sample, followed by centrifugation to remove the plant debris, or make use of direct RNA extraction. Such methods have been used in research studies that investigated the transmission route of virally contaminated water, although in these articles only small portions of fresh produce were sampled (e.g. Esseili et al. 2012b; Hirneisen and Kniel 2013a; Wei et al. 2011). As such, if internalization would be found relevant in certain situations, implemented standard methods contain a lack of methods that are able to detect internal contamination.

Concentration of the eluted viral particles may be achieved by using polyethylene glycol (PEG) as suggested by the ISO/TS 15216-1:2012, or by ultracentrifugation (Kurdziel et al. 2001; Rzeutka et al. 2006), ultrafiltration (Scherer et al. 2010), organic flocculation (El-

Senousy et al. 2013), immunoconcentration (Tian et al. 2011) and cationic separation (e.g. the automated Pathatrix™ separation system (Papafragkou et al. 2008; Stals et al. 2012a). In case of soft red fruits, a matrix prone to cause inhibition in subsequent molecular detection steps, often a further clarification step is required. An organic solvent purification step, using chloroform/butanol to separate the RNA in the aqueous phase, has been suggested in the ISO/TS 15216-1 and in literature (Schrader et al. 2012).

Despite the selection of this elution-concentration method in the ISO/TS 15216-1, this extraction method is far from perfect. Obtained recoveries (0.1% (Mäde et al. 2013) - >40% (Loutreul et al. 2014)) can vary depending on the matrix and the virus under study. As such, obtained mean recovery efficiencies are continuously lower for raspberries when compared with mean recoveries obtained for e.g. lettuce (Scherer et al. 2010; Loutreul et al. 2014). But also high variations in recovery efficiency are observed during screenings in which the food matrix and the virus type (the process control) are kept constant (e.g. recovery efficiency of 41% ± 33% for lettuce in Loutreul et al. 2014). As such, in research they continue to tackle these problems encountered using the current extraction protocols and new research material is continuously published (Coudray et al. 2013; Hida et al. 2013; Shinohara et al. 2013).

VIRUS EXTRACTION FROM ENVIRONMENTAL SAMPLES

Concerning environmental sample analysis, the extraction and concentration methods for detection of enteric viruses from surfaces and hands, and water will be discussed briefly. For the detection of enteric viruses on **surfaces and hands**, swabbing is increasingly being used during outbreak investigations (Boxman et al. 2009) and in environmental research studies (Boxman et al. 2011). Used methods can differ in the type of swab implement used, the type of wetting liquid, the type of eluent and elution/extraction strategy (Julian et al. 2011). As for the implement, different types of swabs (e.g. cotton, polyester, rayon, flocked nylon swabs) and (electrostatic) cloths (e.g. Sodibox swab fabrics, sterile gauze swabs (Smith & Nephew)) have been used for detection of enteric viruses on different surfaces. Strategies used for elution/extraction of viruses from the implement generally comprise mainly of two categories: (i) elution of viruses from swab followed by nucleic acid extraction of the eluent, and (ii) direct RNA extraction from implement. Both strategies are currently applied. Strategy one has been used in the recent VITAL project (e.g. in Maunula et al. 2013; Kokkinos et al. 2012) and strategy two has been proposed by the ISO/TS 15216-1. Despite the implementation of a strict swabbing protocol, recoveries obtained during swabbing studies are characterized by high standard deviations (e.g. range of 1%-38% in Scherer et al. (2009)), even though the surface/swab combination and technician are kept constant (Scherer et al. 2009; Moore and Griffith 2002).

Concerning detection of enteric viruses in **water**, development of proper concentration and detection methods has a much longer history as compared to detection of enteric viruses in food matrices. As such, method development goes back as early as during the 1960s and 1970s (Ikner et al. 2012; Cashdollar and Wymer 2013) (Figure 1.3.). This results in a large body of literature that is available on this topic and a multitude of different concentration strategies that have been developed and implemented the last 60 years.

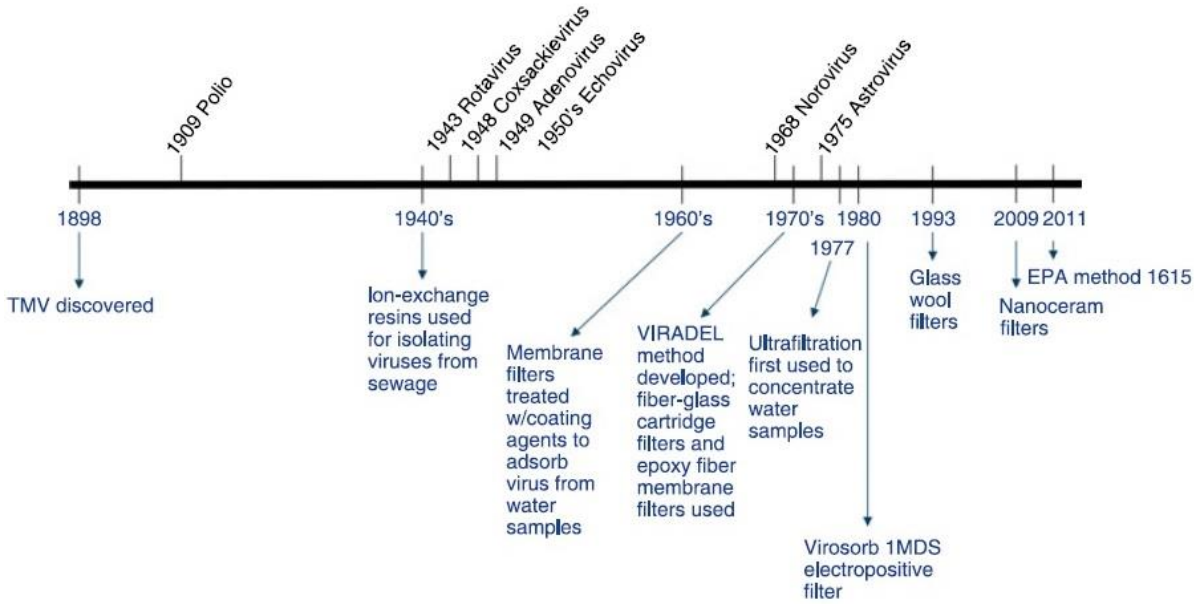


Figure 1.3. History of virus discovery and environmental virology. TMV: Tobacco mosaic virus; VIRADEL methods: virus adsorption/elution methods. Source: Cashdollar and Wymer (2013).

Most of the methods used to concentrate viruses from large volumes of water consist of two concentration steps to be able to concentrate the viruses in an adequate small volume to be used for molecular detection or detection by cell culture. Most primary concentration steps are based on virus adsorption/elution processes, also named VIRADEL. This method involves the adsorption of viral particles to the filter media by charge interaction and subsequent elution of the virus by a pH-adjusted solution. Because viruses in water typically have a net negative charge (isoelectric point (pI) of NoV and HAV is 5.5-6.0 and 2.8 respectively (Michen and Graule 2010)), viruses can adsorb to electropositive filters by electrostatic interactions. As such the two basic filters used are electropositive filters and electronegative filters, either in the format of cartridges or flat membrane filters. However in the case of the use of an electronegative filter, either the filter or the water sample needs conditioning to allow virus adsorption. Also glass wool matrices (electropositive charged) are used as filter media (e.g. Lambertini et al. 2008; Wyn-Jones et al. 2011). Another primary concentration method increasingly researched the last decade, is ultrafiltration. This method is based on size exclusion rather than charge interaction and has also been

used for large volumes of water, e.g. 100 L groundwater samples by Olszewski et al. (2005).

Commonly used secondary concentration steps are organic flocculation by e.g. acidifying the beef extract eluate to stimulate the formation of a virus-protein floc which then can be pelleted by centrifugation and redissolved in a smaller volume, and ultrafiltraton by use of small centrifugal ultrafilters such as e.g. Vivaspin (Sartorius-Stedim) (Cashdollar et al. 2013), Centricon centrifugal ultrafilters (Millipore) (Soto-Beltran et al. 2013).

Several combinations of primary and secondary concentration steps are currently in use. As such, both Health Canada (Simard et al. 2007) and the recently published ISO/TS 15216-1 propose the use of a positively charged membrane filter in combination with the use of a centrifugal filter concentration device as secondary concentration method for the detection of HAV and NoV in bottled water. In the recent European VITAL project water samples were analyzed using electropositive glass wool in combination with organic flocculation (e.g. in Maunula et al. 2013). The recently developed Method 1615 by the US EPA for measurement of enterovirus and norovirus occurrence in water by culture and RT-qPCR comprises the use of the electropositive NanoCeram cartridge filter (Argonide) or the 1MDS Virosorb cartridge filter (Cuno), with organic flocculation as secondary concentration method and even includes a tertiary concentration in case of detection by RT-qPCR using the Vivaspin ultrafilters (Sartorius-Stedim) (Fout et al. 2010).

For further information on these concentration methods and their obtained recovery efficiencies and other concentration strategies, extensive reviews for detection of enteric viruses in water are available in literature (Gensberger and Kostic 2013; Fong and Lipp 2005; Cashdollar and Wymer 2013; Ikner et al. 2012; Wyn-Jones and Sellwood 2001; Mattison and Bidawid 2009).

1.1.3.2. Detection methods

NoV are difficult to study because until now no practical cell culture assay or animal model is available for virus production and quantification (Duizer et al. 2004). However recently advances have been made in this area by Jones et al. (2014) who developed a cell culture system for human NoV (GII.4-Sydney human NoV). NoV were shown to infect human B cells and HBGA-expressing bacteria were identified as a potential cofactor to facilitate productive attachment of the virus to the epithelial cells and hence to facilitate infection of the B cells. Nevertheless further research is needed before practical implementation can be even considered. Also for HAV an efficient cell culture for detection of wild-type strains is lacking (Goyal 2006). Hence, for detection **molecular amplification based methods** are needed, as expected concentrations in foods are too low

for use of e.g. electron microscopy (EM) and enzyme immunoassays as mentioned earlier for detection in feces samples. Generally, the molecular detection strategy consists of two step: (i) first an RNA extraction and purification step, followed by (ii) the molecular detection step.

The most popular RNA extraction methodology is based on the chaotropic agent guanidine thiocyanate combined with a purification method based on the adsorption to silica to assist purification through several washing stages. This methodology is also proposed by ISO/TS 15216-1 and several kits are available on the market that use this strategy (e.g. NucliSens® easyMAG™ and NucliSens MiniMAG (Biomérieux), RNeasy Mini Kit (Qiagen)). The removal of inhibitory compounds is a crucial step because the reverse transcriptase step and the PCR is highly susceptible to food-derived inhibitors (Girard et al. 2013).

The most popular molecular detection method is reverse transcriptase polymerase chain reaction (RT-PCR), whether or not in real-time format (RT-qPCR). RT-qPCR is considered to be the gold standard for detection of NoV in clinical, food and environmental samples (Baert et al. 2007; Jothikumar et al. 2005). As in comparison to the conventional RT-PCR, this assay results in a lower detection limit, does not require post-PCR processing, and enables quantification of genomic copies. Generally human NoV belonging to GI and GII are targeted during screening, each requiring separate conservative primer pairs. For more information on the use of molecular detection for detection of foodborne enteric viruses, reviews are available (Mattison and Bidawid 2009; Stals et al. 2012b).

The two main **drawbacks** for use of the currently implemented detection protocols are: **(i)** the *limited sample volume* that can be assayed using these molecular methods (normally 3 – 10 µl), while in contrast to bacterial detection, enrichment is not possible. This stresses the importance of the availability of an efficient extraction and concentration method for detection of NoV in different matrices in order to be able to analyze representable sample sizes. The most important drawback **(ii)** is the inability of the currently used RT-qPCR method to differentiate between RNA detected from infectious viral particles, defective viruses and free RNA. Molecular methods only indicate the presence or absence of genomes or fragments of genomes of the target virus in a given sample and do not provide any information on its *infectivity* and hence associated health risk. As for a viral particle to be infective, both an intact viral capsid and intact viral genome are required (Figure 1.4.). Therefore, when viral RNA is detected the related health risk is not evident as infectivity could be lost by impairment of the capsid or incompleteness of the rest of the RNA genome outside the targeted section used for RT-qPCR detection. This further implicates the assessment of the results.

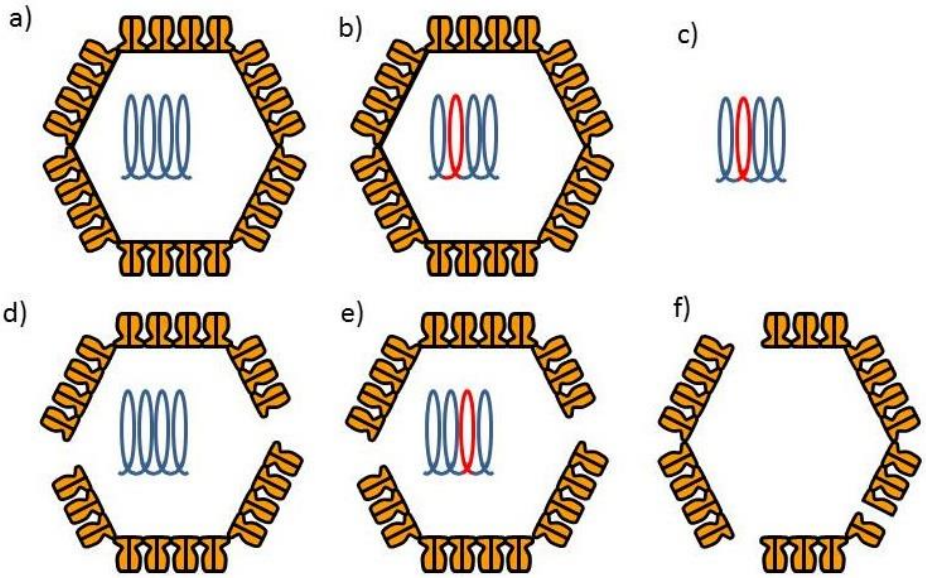


Figure 1.4. Different virus particles and remnants present in the environment presented as simplified schematics of NoV particles (protein capsid and viral RNA genome), whether or not including impairments of capsid and/or viral RNA (visualized in red). Only one figure (i.e. a) represents an infectious NoV particle, but all versions can be present in the environment.

1.1.3.3. Available data for foodborne viruses in fresh produce in literature and considerations

In literature only limited screening studies are available for foodborne viruses (HAV and NoV) in fresh produce, this in contrast to the numerous screening studies available for NoV and HAV in shellfish (e.g. Manso and Romalde 2013; Benabbes et al. 2013) and in water (i.e. (reclaimed) wastewaters, surface waters, e.g. in Lee et al. 2014; Mans et al. 2013).

In Table 1.2., peer-reviewed screening studies are listed for foodborne viruses NoV and HAV in fresh produce not implicated in outbreaks, and not part of specifically designed transmission route studies (e.g. strawberries irrigated with river water in Brassard et al. 2012). Studies taken up in Table 1.2. were further restricted to those that analyzed at least 20 samples of at least one fresh produce commodity under consideration and used molecular detection methods.

Keep in mind that detected prevalence is influenced by the practical limit of detection (LOD) (also influenced by recovery efficiency of the elution/concentration method) of the used detection strategy. This implies that if the recovery efficiency of the used detection strategy is low, there is a smaller chance of detecting viruses in the produce. As such in the study of Brandao et al. (2014) on GII NoV prevalence in lettuce in Brazil none of the 90 lettuce samples were positive. However, when evaluating the detection strategy for detection of the process control virus (i.e. PP7 bacteriophage) and GII NoV a recovery

efficiency of <1% was obtained for all tested samples (n=9) and not all inoculated samples with GII NoV tested positive (merely 6/9). This indicates that the implemented adsorption-elution concentration method was not that efficient or inhibition of the RT-qPCR step played a significant role (no internal amplification control was included). Also the detection of the process control during the screening was inadequate as indicated in Table 1.2. In conclusion, harmonizing the data from reported studies is difficult due to differences in the sensitivities of the detection methodologies employed (Baert et al. 2011).

Remark that next to the issue of an insufficient LOD, also the presence of inhibitors co-extracted from the sample matrix can lead to under-estimation of the prevalence of enteric viruses in food. As such, next to knowledge on the LOD, the inclusion of proper inhibition controls is crucial for correct interpretation and assessment of the results. However not all prevalence studies in Table 1.2. included this.

Overall 0% to 40% of soft red fruits and 0% to 50% of leafy green vegetables tested positive for NoV. The presence of GI NoV clearly dominated the prevalence data for vegetables outlined in Table 1.2. As such in two large studies performed in France (Loutreul et al. 2014) and Egypt (El-Senousy et al. 2013) over 96% of NoV positives was attributed to the presence of GI NoV. Even though GII NoV was also detected in the irrigation waters analyzed for the Egyptian study. This is in accordance to the observation of Matthews et al. (2012), as despite the general predominance of GII NoV in NoV outbreaks, a significant smaller proportion of waterborne and foodborne outbreaks was associated with GII strains compared to other modes of transmission. In foodborne and waterborne outbreaks the association with multiple (GI + GII) NoV strains (e.g. in Stals et al. 2011b and Mattison et al. 2010) is also more common than in person-to-person and environmental outbreaks (Matthews et al. 2012). The relative higher prevalence of GI NoV in vegetables and in FBO may be explained by a greater persistence of NoV GI compared to NoV GII as hypothesized by e.g. Butot et al. (2009) and da Silva et al. (2007).

Concerning the prevalence of HAV, only six screening studies were available, and in only one study, located in a high endemic region, HAV was detected in fresh produce. More prevalence data for NoV and HAV presence in fresh produce is requested for adequate risk assessments concerning the viral risk attributed to the consumption of fresh produce.

Table 1.2. Available prevalence data on NoV and HAV in soft red fruits and leafy greens in peer-reviewed literature.

Study **Country** **Sampling place** **Virus under study**	**Commodity**	**# positive samples/ # samples**	**% of positive samples [95% CI]**[a]	**Numbers of genomic copies in positive samples** (range or mean)
Hernandez et al. 1997[c] Costa Rica Point of sale (farmer markets) HAV	Lettuce (n=50 samples, pools of 5 lettuce samples)	2/10 pools HAV positive	4.0% - 20.0%	NQ
De Giusti et al. 2010[c] Italy Producer/Company NoV (GI & GII) and HAV	Endive (whole)	0/16	0.0% [0.0, 19.4]	NA
	Curly endive (whole)	0/10	0.0% [0.0, 27.8]	NA
	Rucola lettuce (whole)	0/10	0.0% [0.0, 27.8]	NA
	Red chicory (whole)	0/10	0.0% [0.0, 27.8]	NA
	Mixed salads (RTE)	0/30	0.0% [0.0, 11.4]	NA
	Endive (RTE)	0/24	0.0% [0.0, 13.8]	NA
	Rucola lettuce (RTE)	0/24	0.0% [0.0, 13.8]	NA
Mattison et al. 2010; Baert et al. 2011 Canada Retail NoV (GI & GII)	Packaged leafy greens	133/641 GI, 106/641 GII =>181/641	28.2% [24.9, 31.9]	1.0 – 8.3 log_{10}/g
Stals et al. 2011b Belgium Company NoV (GI & GII)	Raspberries	3/10 GI, 3/10 GII => 4/10	40.0% [16.8, 68.7]	2.45 – 3.70 log_{10}/10g
	Cherry tomatoes	5/30 GI, 4/30 GII => 7/30	23.3% [11.8, 40.9]	4.07 – 5.04 log_{10}/10g
	Strawberries	4/20 GI, 3/20 GII => 6/20	30.0% [14.6, 52.0]	2.29 – 4.10 log_{10}/10g
	Mixed-fruit salad	0/15 GI, 1/15 GII => 1/15	6.7% [1.2, 29.8]	4.64 log_{10}/10g
Baert et al. 2011 France & Belgium Company NoV (GI & GII)	Raspberries (n=142) and strawberries (n=8) (France)	3/150 GI, 9/150 GII => 10/150	6.7% [3.7, 11.8]	2.4 – 5.8 log_{10}/g
	Leafy greens (France)	2/6 GI, 1/6 GII => 3/6	50% [18.8, 81.2]	2.0 – 3.5 log_{10}/g
	Leafy greens (Belgium)	2/6 GI, 0/6 GII => 2/6	33.3% [9.7, 70.0]	1.9 – 3.1 log_{10}/g
Yilmaz et al. 2011[c] Turkey Restaurants NoV (GI & GII)	Lettuce	0/98 GI, 0/98 GII => 0/98	0.0% [0.0, 3.8]	NA
	Parsley	0/92 GI, 0/92 GII => 0/92	0.0% [0.0, 4.0]	NA
	Mixed salad	0/65 GI, 0/65 GII => 0/65	0.0% [0.0, 5.6]	NA
	Green onion	0/92 GI, 1/92 GII => 1/92	1.1% [0.2, 5.9]	NQ
	Tomatoes	0/95 GI, 1/95 GII => 1/95	1.1% [0.2, 5.7]	NQ
Serracca et al. 2012[c] Italy Point of sale NoV (GI & GII) and HAV	Packaged leafy greens	0/80 GI, 0/80 GII, 0/80 HAV => 0/80	0.0% [0.0, 4.6]	NA

Kokkinos et al. 2012	Fresh lettuce	2/149 GI	1.3% [0.4, 4.8]	5 PDU per 25 g[b]
Greece, Serbia, Poland		1/126 GII	0.8% [0.1, 4.4]	10 PDU per 25 g[b]
Point of sale		0/149 HAV	0.0% [0.0, 2.5]	NA
NoV (GI & GII) and HAV				
Maunula et al. 2013	Raspberries (fresh)	0/60 GI, 0/60 GII, 0/60 HAV => 0/60	0.0% [0.0, 6.0]	NA
Czech Republic, Poland, Serbia	Raspberries (frozen)	0/39 GI, 0/39 GII, 0/39 HAV => 0/39	0.0% [0.0, 9.0]	NA
Point of sale	Strawberries (fresh)	0/21 GI, 0/21 GII, 0/21 HAV => 0/21	0.0% [0.0, 15.5]	NA
NoV (GI & GII) and HAV				
El-Senousy et al. 2013[e]	Lettuce	35/144 GI, 0/144 GII => 35/144	24.3% [18.0, 31.9]	$6.3 \times 10^2 \pm 1.8 \times 10^2$ /g
Egypt (Nile delta)	Watercress	45/144 GI, 0/144 GII => 45/144	31.3% [24.3, 39.2]	$5.2 \times 10^2 \pm 2.5 \times 10^2$ /g
Farm level	Radish	37/144 GI, 0/144 GII => 37/144	25.7% [19.3, 33.4]	$1.7 \times 10^2 \pm 3.4 \times 10^1$ /g
NoV (GI & GII)	Leek	30/144 GI, 0/144 GII => 30/144	20.8% [15.0, 28.2]	$5.9 \times 10^2 \pm 1.1 \times 10^2$ /g
	Green onion	49/144 GI, 0/144 GII => 49/144	34.0% [26.8, 42.1]	$5.6 \times 10^2 \pm 2.7 \times 10^2$ /g
Perez-Rodriguez et al. 2014	Unprocessed lettuce	2/30 GI, 2/30 GII, 0/30 HAV	/	NQ
Spain	RTE lettuce	0/30 GI, 2/30 GII, 0/30 HAV => 2/30	6.7% [1.9, 21.3]	NQ
Processing level	⇨ 5 samples/lot			
NoV (GI & GII) and HAV	⇨ 30 lots			
Brandao et al. 2014	Whole fresh lettuce	0/30[d]	0.0% [0.0, 11.4]	NA
Brazil	Minimally processed lettuce	0/30[d]	0.0% [0.0, 11.4]	NA
Point of sale	RTE lettuce salad	0/30[d]	0.0% [0.0, 11.4]	NA
GII NoV				
Loutreul et al. 2014	Chicory (unwashed)	12/107 GI, 1/107 GII => 13/107	12.1% [7.2, 19.7]	NQ
France	Lettuce (unwashed)	10/77 GI, 0/77 GII => 10/77	13.0% [7.2, 22.3]	NQ
Company	Lamb's lettuce (unwashed)	3/26 GI, 0/26 GII => 3/26	11.5% [4.0, 29.0]	NQ
NoV (GI & GII)	Raspberries (frozen)	27/162 GI, 0/162 GII => 27/162	16.7% [11.7, 23.2]	NQ
	Strawberries (frozen)	3/32 GI, 1/32 GII => 4/32	12.5% [5.0, 28.1]	NQ
	Blackberries (frozen)	1/2 GI, 0/2 GII => 1/2	50.0% [9.5, 90.6]	NQ
	Mixed berries (frozen)	0/4 GI, 0/4 GII => 0/4	0.0% [0.0, 49.0]	NQ

NA: not applicable; NQ: no quantification was done; CI: confidence interval; RTE: ready-to-eat; whole: unprocessed, sampled 'raw' crops

[a]: 95% CI was calculated using the method of Wilson with no continuity correction using an online application (http://vassarstats.net/prop1.html)

[b]: in this study, instead of using a calibrated quantitative assay a most probable number approach was used using end-point detection of RT-PCR signal in dilutions of nucleic acid extracted from the sample, and therefore the data are expressed as 'PCR-detectable units' (PDU).

[c]: Did not include any process control/inhibition control

[d]: Inherent low recovery efficiency of implemented extraction method observed during testing and unsatisfactory results obtained using PP7 bacteriophage as process control virus during analysis, can lead to false negative results. Giving rise to doubts on the relevance of these results. Recovery success rate of the PC was 12/30, 26/30, and 23/30 for whole fresh lettuce, minimally processed lettuce, and RTE lettuce salad respectively. Due to low recovery efficiency and/or inhibition during the RT-qPCR step.

[e]: Raw NoV genome copy numbers measured by RT-qPCR, in duplicate, were corrected according to virus/nucleic acid extraction and RT-PCR efficiencies.

1.2. TRANSMISSION ROUTES OF NOROVIRUS RELEVANT FOR FRESH PRODUCE AND ENVIRONMENTAL PERSISTENCE

Direct person-to-person contact, including transmission by aerosolized fomites, is the primary transmission route of enteric viruses such as NoV and hence responsible for the majority of outbreaks. Estimates of human NoV outbreaks due to person-to-person spread range from 69% to 85% in reports from the USA (Hall et al. 2014), the Food Borne Virus European network (FBVE) (Verhoef et al. 2010), the UK (Lopman et al. 2003), and The Netherlands (Siebenga et al. 2007). Besides this, identified indirect transmission routes are contaminated water or soil, contaminated food or contact surfaces, as illustrated in Figure 1.5. Foodborne transmission is estimated to be responsible for approximately 22% and 23% of NoV outbreaks in Europe (Verhoef et al. 2009) and the USA (Hall et al. 2014) respectively. In this literature overview the focus is on fresh produce as a vehicle for foodborne norovirus outbreaks, and more specific leafy greens (e.g. lettuce) and soft red fruits (e.g. raspberries), which have been repeatedly linked with viral FBO as was illustrated in outbreak Table 1.1.

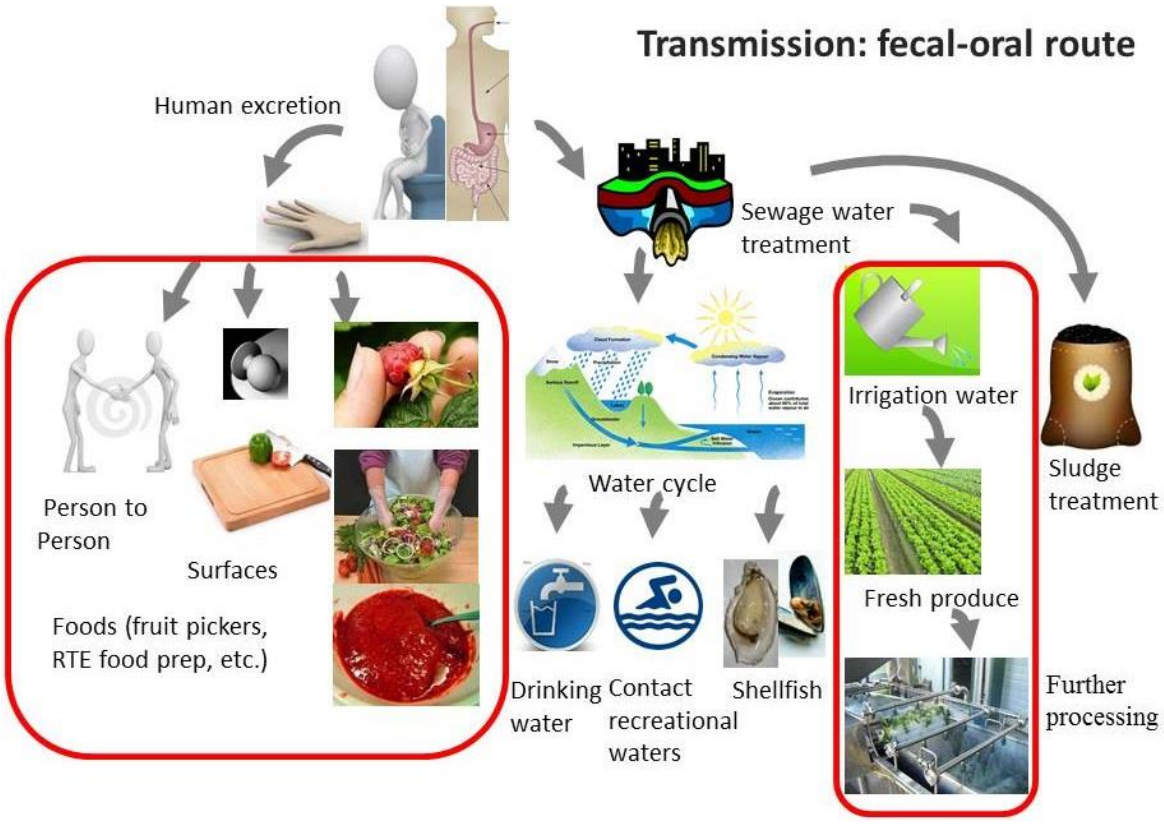


Figure 1.5. Schematic overview of the fecal-oral transmission route of human infective NoVs. Source: Personal communication with dr. Li Dan (2014).

Viral transfer to fresh produce may be divided into two phases: contamination taking place pre-harvest and contamination taking place during harvest or further post-harvest processing. Enteric viruses such as NoV and HAV follow the fecal-oral transmission route, and NoV can also be transmitted through aerosolized vomitus. A person infected with NoV can shed up to 12 $\log_{10}$ viruses (RT-PCR) per g feces (Atmar et al. 2008) and $>10^6$ NoV particles (EM) per ml vomitus (Caul 1994). A person infected with HAV can excrete 10^6-10^8 particles/g of feces during infection (Sanchez 2013).

As both human NoV and HAV are currently believed to be non-zoonotic viral pathogens, their sole host and means of replication are human cells and hence the primary cause of contamination is contact with (residue of) infected and shedding people. Next to the high viral load during shedding and the low infectious dose, also environmental persistence facilitates water- and foodborne spread of enteric viruses such as NoV and HAV. Hence in this section, next to the casus of the contamination of fresh produce, also environmental persistence will be included during each of the stages of the farm-to-fork production chain. Both knowledge on transmission routes and persistence are key to identify possible prevention and control efforts for risk mitigation.

In Figure 1.6. a schematic depiction of a risk assessment framework is given for the presence of NoV in the fresh produce chain. This framework illustrates the possible sources of contamination of NoV input(s) from farm-to-fork chain and the influencing factors that could affect the viral persistence and thus the potential risk to the consumer.

1.2.1. Pre-harvest contamination

In the following paragraphs the persistence and transmission by means of contaminated seeds, soil, and water at the pre-harvest level will be discussed more in detail, ending with a paragraph on the persistence of enteric viruses on the crops in the field.

1.2.1.1. Contaminated seeds

The life cycle of plants starts with a seed. Contaminated seeds have been identified as a major cause for outbreaks with sprouted seeds (Yang et al. 2013): e.g. the large European outbreak in 2011 associated with fenugreek seeds contaminated by *Escherichia coli* O104:H4 (Buchholz et al. 2011). In all these outbreaks foodborne bacteria, such as various serotypes of *Salmonella* and *E. coli* O157:H7, have been identified as the cause (Yang et al. 2013; Harris et al. 2003).

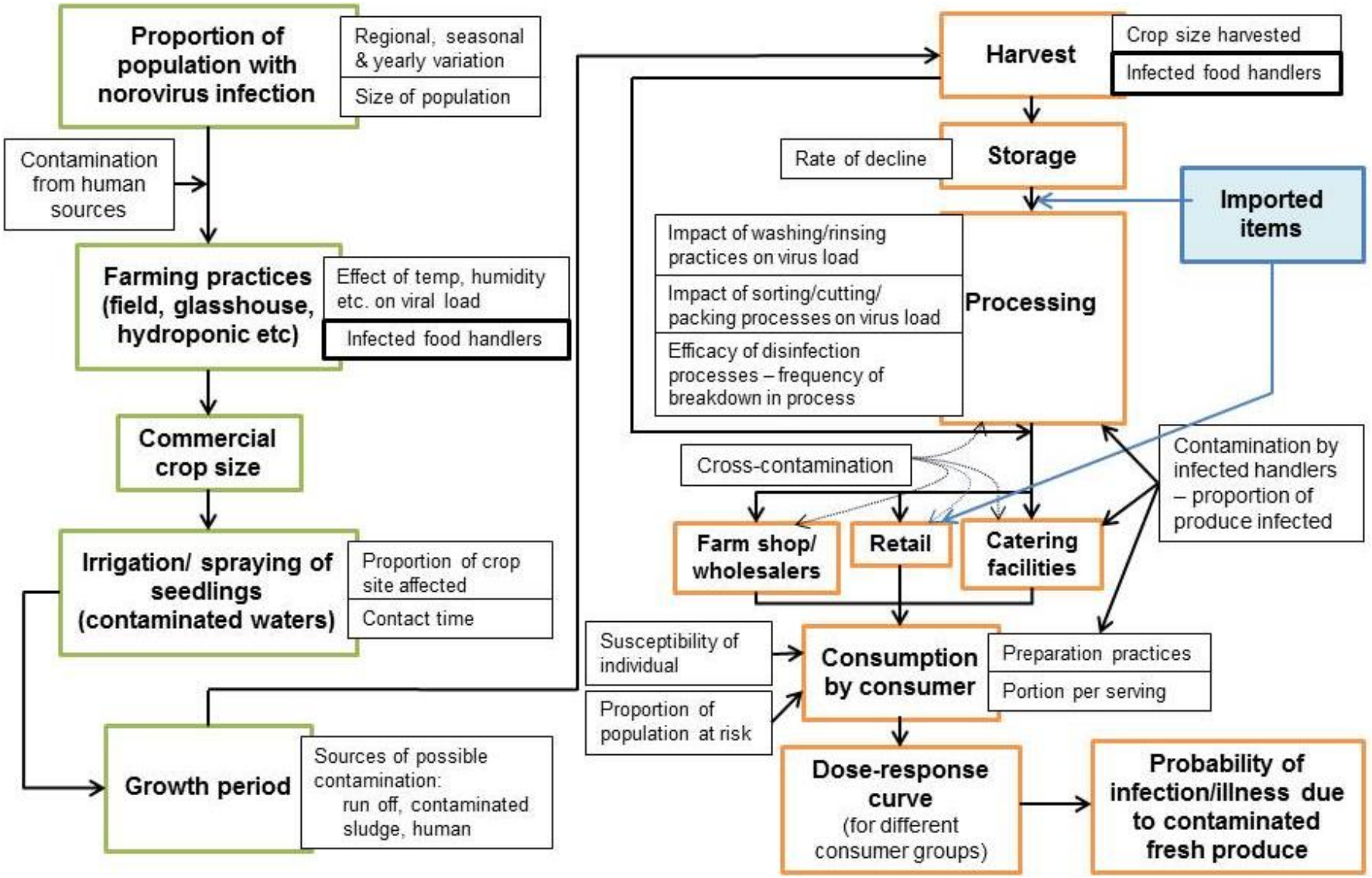


Figure 1.6. Basic risk flow framework addressing sources of NoV input(s) from farm-to-fork and elements that could affect viral persistence in the fresh produce chain. Adapted from Lawrence et al. (2004).

However viral outbreaks have not yet been linked to sprouted seeds and therefore bacterial pathogens were the subject in multiple studies conducted to better understand the interaction of the pathogens with seeds and resulting sprouts (Castro-Rosas and Escartin 2000; Gomez-Aldapa et al. 2013). Nevertheless, to understand whether viral contaminated seeds could also pose a threat to human health, Wang et al. (2013a) investigated the persistence of HAV and human NoV surrogates MNV and TV on alfalfa seeds during storage and on sprouts after a 7-day germination period (Wang et al. 2013b). During this study it was concluded that HAV, and surrogates MNV and TV could persist for a prolonged period on the surface of the alfalfa seeds as the viruses remained infectious after 50 days. Following a 7-day germination period, viruses were located in all tissues as well as in sprout-spent water sampled on several occasions during germination (Wang et al. 2013b). As such good agricultural practices (GAP) during production of seeds, possibly followed by postharvest intervention measures, and appropriate control measures to prevent cross-contamination due to reuse of water during germination should also focus on enteric viruses as a possible contaminant.

1.2.1.2. Contaminated soil, contaminated manure or sludge

Another route of contamination for fresh produce is soil. Although specific FBOs due to fresh produce linked to viral contaminated soil are missing, proof of concept has been demonstrated in a study by Wei et al. (2010). Attachment of a NoV surrogate (MNV-1) was observed upon contact of lettuce with spiked treated sludge and manure. Hence viral presence in soil due to soil amendment with these matrices may increase the risk of fresh produce contamination (Wei et al. 2010). Experimental field studies on transfer of bacterial pathogens onto fresh produce due to amended soils are present in literature (e.g. Ongeng et al. 2011).

Contaminated soil, manure and sludge implies a risk of introducing pathogens on vegetables and other crops either directly through direct soil-plant contact, soil splashing due to rain or irrigation, or due to formation of aerosols/dust, or indirectly via contamination of irrigation water, run-off from or flooding of neighboring surroundings. As the human enteric viruses under study, NoV and HAV, are generally strictly confined to humans as their sole hosts, unlike for some bacterial pathogens, application of cow dung or manure of other animals to the soil as fertilizer does not contribute to viral contamination of the produce. However application of manure or slurry *contaminated* with excrements of human origin, or the proximity of a latrine may pose a risk. A second source of viral contamination of the soil is the application of sludge. Sludge originates from the process of waste water treatment and hence might contain next to heavy metals high loads of pathogens (viruses, bacteria etc.) present in waste waters. Sludge might be applied to

agricultural lands to enrich the soil with valuable organic matter and nutrients. Usually the sewage sludge should have been subjected to treatment methods that are intended to reduce the number of pathogens while retaining beneficial properties for fertilization and other soil amendment and land reclamation purposes (US EPA 2011).

There are strict requirements for the use and application of sewage sludge on farmland where crops such as salads and vegetables are grown. As such, directive 86/278/EEC states that sludge shall be treated before use in agriculture. EU member states can reinforce specific treatment requirement. For example in the UK only the use of enhanced treated sludge (~biosolids) is allowed for fresh produce crops, defining enhanced treatment as a treatment process capable of ensuring a 6 $\log_{10}$ reduction in pathogens (BRC et al. 2001). However a 6 log reduction of enteric viruses can be challenging as three of the most common treatment techniques for sewage sludge reviewed in Viau et al. (2011) (i.e. mesophilic anaerobic digestion, temperature-phased/thermophilic anaerobic digestion, and composting) resulted only in mean reductions ranging from 1.3 to 2.4 $\log_{10}$ PFU for enteroviruses and adenoviruses. A second requirement is the instalment of a 10 month harvest interval between application of the sludge and harvest of these fresh produce crops (~withholding period) (Council Directive 86/278/EEC).

These high requirements are not met around the world, as the application of untreated sludge or poorly treated sludge is still common practice in several regions of the world (Levasseur et al. 2007). It is assumed that enteric virus numbers in sludge would at least be similar, probably greater than, those reported in wastewater. Although exact data on **presence** and concentration of enteric viruses in sludge is largely restricted to enteroviruses (10^2 to 10^4 PFU g^{-1} dry weight in raw sludge) (Sidhu and Toze 2009), some data confirming the possible presence of HAV, NoV, AdV and RV are available (Schlindwein et al. 2010; Wei and Kniel 2010; Prado et al. 2014).

Concerning the **persistence** of enteric viruses in soil (amended with treated sludge, manure or feces), temperature and moisture are primary factors influencing persistence. Nonetheless, persistence was also found to be dependent on the virus-type and soil type/conditions (e.g. pH) (Hurst et al. 1980). Microorganisms tend also to survive longer in sub-surface soil as the soil surface is generally harsher to microbial survival because of desiccation and exposure to solar radiation (Song et al. 2006). Overall, both relatively short term (e.g. 11 days persistence of poliovirus in soil in Ohio in summer (Tierney et al. 1977)) and long term persistence (e.g. ≥6 month persistence of coxsackievirus in soil in winter in Denmark (Damgaardlarsen et al. 1977)) of enteric viruses in (amended) soils have been observed. More detailed reviews on persistence in (amended) soil, feces and/or biosolids are available in literature (Rzezutka and Cook 2004; Wei and Kniel 2010).

1.2.1.3. Contaminated water

Water has been identified as another environmental route of contamination of fresh produce next to soil (Steele and Odumeru 2004; Pachepsky et al. 2011). Intentional application of water at the farm stage includes the use of water for irrigation, the use of water to dissolve and apply chemicals (e.g. fungicide, pesticide, insecticide) to the produce and the use of water for cleaning of equipment. In this paragraph proof of concept, general description of the transmission route by irrigation water and influencing factors, presence and persistence of enteric viruses in relevant water matrices, route of contamination of water, and influence of extreme water-related weather events will be discussed successively.

PROOF OF CONCEPT

Proof of concept for contaminated irrigation and spray water as relevant transmission route for enteric viruses is available in literature. As such, links to a major outbreak in Czech Republic in 1979 (28 880 ill persons) specifically linked to HAV contaminated frozen strawberries due to wrongful irrigation with sewage can be found (LeggeÄ 1997; Vasickova et al. 2005). However viral FBO due to possible contamination as a result of vegetable or fruit spraying with insecticides and fungicides mixed with contaminated water were not found in literature. Nevertheless this route of contamination has been suspected as the cause of a FBO due to raspberries contaminated with *Cyclospora cayetanensis* in the USA (1996) (CDC 1996; Herwaldt et al. 1997; Palumbo et al. 2013). Besides, the relevance of these two transmission routes for viral pathogens has been proven during experimental field studies (Brassard et al. 2012; Cheong et al. 2009) and by the use of QMRA (Stine et al. 2011; Stine et al. 2005b).

GENERAL DESCRIPTION OF THE TRANSMISSION ROUTE BY IRRIGATION WATER AND INFLUENCING FACTORS

Viral contamination of plants by means of irrigation water may occur in **two ways**, either by direct contact like by spray or splash, or through internalization into the tissue via the root system of the plant. However a recent review on internalization of human enteric pathogens (both bacteria and viruses) considers the risk of root uptake of pathogens into produce through the roots via contaminated *soil* (e.g. by irrigation) as relatively low (Hirneisen et al. 2012). Nevertheless, for studies in which plants are grown in viral contaminated *hydroponic solution*, high contamination levels of enteric viruses in edible plant tissue have been demonstrated, e.g. contamination levels exceeding 4 $\log_{10}$ GC/plant for HAV and MNV-1 have been found in all portions of both green onion and spinach plants, including the edible portions (Hirneisen and Kniel 2013a). Transpiration is the driving force for water absorption, and the majority (96%) of water is taken up by the plant through transpiration. As such transpiration has been suggested as the major force for virus

uptake through roots (Wei et al. 2011) and hence uptake can be described as passive. Similar as for bacteria, direct contact of water with produce (e.g. spray, washing) can also lead to internalization or physical entrapment of viruses in lettuce via both the stomata and cut edges and hence protecting viruses from washing and sanitation (Wei et al. 2010). In contrast to bacteria, viruses are considered non-living when they are outside their hosts and consequently the attachment of viruses to stomata or to a cut edge is a matter of probability rather than preference as viruses cannot move to specific locations on the plant epidermis (Wei et al. 2010; Hirneisen and Kniel 2013c). Nonetheless, viral attachment/adsorption to food surfaces can depend on virus-specific factors (e.g. virus isoelectric point (pI), presence of food-specific ligands), food (surface) factors (e.g. presence of virus-specific ligands, access to food interior) (Esseili et al. 2012a) and extrinsic factors (e.g. pH and presence of substances competing for binding) (Vega et al. 2005; Le Guyader and Atmar 2008). For example, using confocal microscopy Gandhi et al. (2010) observed that recombinant norovirus-like particles localized in clusters on the veins of romaine leafs rather than with an even distribution throughout the romaine leaf, implying that binding may involve a specific ligand(s) on the leaf surface.

Transfer of organisms from water to produce surfaces via irrigation is **influenced by** irrigation method (Alum et al. 2011; Song et al. 2006) and the type of produce (e.g. soil-effluent-plant contact situations and surface properties) (Bastos et al. 2008). Irrigation method is an important factor as choosing an optimal strategy can minimize the contact of irrigation water with the aboveground portion of the crop and hence lower the risk of viral contamination. As such, in a field study by Stine et al. (2005b) no viral contamination of lettuce was detected when grown using sub-surface drip irrigation practices, while the use of furrow irrigation led to contaminated lettuce. Other commonly used irrigation systems are sprinkler irrigation, and surface drip (Wei and Kniel 2010). Crops irrigated with sprinkler and furrow systems may have a higher chance of direct contact with viruses and are hence considered to be more hazardous for fresh produce crops such as lettuce (Wei and Kniel 2010). Remark that transfer from contaminated water to fresh produce can also occur through aerosols formed e.g. during sprinkler irrigation and viruses have the characteristic to be transferred more easily and over a larger area compared to certain indicator bacteria (e.g. coliforms) (Teltsch et al. 1980). As such, several studies have identified subsurface drip irrigation as a tool to mitigate microbial risks of crops (Alum et al. 2011; Song et al. 2006). However non-favorable soil and field conditions such as the use of a shallow drip tape installation and preferential water paths through cracks on the soil surface are suggested to be able to lead to viral contamination in sub-surface drip irrigation plots (Choi et al. 2004). Concerning the influence of produce type, leafy vegetables such as lettuce, with high water retention capacity and in close contact to the

ground, are identified to be especially vulnerable to viral contamination through irrigation (Hamilton et al. 2006). High growing plants such as bell peppers show lower microbial contamination levels when irrigated at soil level compared to low growing crops such as lettuce (Bastos et al. 2008). As such, both furrow irrigation and subsurface irrigation did not lead to viral contamination of bell peppers during field studies (Stine et al. 2005b; Song et al. 2006). Nevertheless in a study by Alum et al. (2011) contamination of tomato fruits and cucumber fruits occurred using surface drip irrigation, while no viruses were detected on the fruits when subsurface drip irrigation was used.

PRESENCE AND PERSISTENCE OF ENTERIC VIRUSES IN RELEVANT WATER MATRICES

Presence of enteric viruses has been demonstrated in all sorts of waters generally used for irrigation of produce. As such NoV have been detected e.g. in ground water wells in the USA (Fout et al. 2003), Korea (Cheong et al. 2009; Park et al. 2010b) and Italy (Gabrieli et al. 2009); in canal waters in the USA (Kayed 2004); in reclaimed wastewater, and in river water samples all over the world (Poland (Kozyra et al. 2011), the Netherlands (Westrell et al. 2006), Japan (Haramoto et al. 2005), South-Africa (Mans et al. 2013)). For instance in water from the Nile Delta, used for irrigation of fresh produce in Egypt, concentration around 10^2 genomic copies per liter were found for both GI and GII NoV (El-Senousy et al. 2013). Sources of irrigation water can be generally ranked by the microbial contamination hazard: in order of increasing risk these are potable or rain water, groundwater from deep wells, groundwater from shallow wells, surface water, and finally raw or inadequately treated wastewater (Pachepsky et al. 2011).

The omnipresence of enteric viruses in these waters can be explained by (i) the recalcitrance of enteric viruses such as noroviruses towards wastewater treatments as viruses have been detected in both influent and effluent waters (Battistone et al. 2014; da Silva et al. 2007; Sidhu and Toze 2009), (ii) the lack or deficient state of current sewage systems and the omnipresence of viral contamination sources such as leaking septic tanks, latrines, combined with a higher potential for transport in soil compared to e.g. bacteria (Hijnen et al. 2005), and (iii) the high persistence of enteric viruses in these waters. As such, it is demonstrated that NoV (GI.1) can remain infectious for at least two months in groundwater (dark, RT) and can remain detectable over three years (Seitz et al. 2011). In case of human AdV, long-term **survival studies** indicated persistence in ground water over a year (Charles et al. 2009). In general, mean inactivation rates of viruses in fresh water are less than 1 $\log_{10}$ per day, indicating that viruses can persist in freshwater sources for prolonged periods of time (Rzezutka and Cook 2004). Persistence of surrogate virus MNV-1 has also been observed in reconstituted pesticides (Verhaelen et al. 2013b). PCR detection has often been observed to overestimate the viral risks due to detection of

infectious and defective particles. With time, the ratio of infectious particles to genomic copies (molecular detection) has been observed to decrease and as such this ratio is partly depending on the 'age' of contamination (De Roda Husman et al. 2009). The persistence of enteric viruses in water is known to be affected by temperature, virus association with solids, exposure to light (UV), and the presence of indigenous microbiota. These are all factors that are known to be substantially different from one geographical location to another (Bosch et al. 2006; John and Rose 2005).

ROUTE OF CONTAMINATION OF WATER

The main route of contamination of surface waters is by sewage and contaminated effluent waters of ineffective wastewater treatment plants (WWTP). Factors controlling the occurrence of viruses in surface waters are type of sewage treatment in place, type of disinfectant, time of the year, incidence of infection in the community and precipitation (Gerba 2007). Sources of viruses in groundwater are leaking septic tanks and sewer lines, unlined landfills (e.g. diaper disposal), wastewater irrigation or land application of sludge, subsurface injection of wastewater, and (infiltration from) nearby contaminated surface water (Gerba 2007; Borchardt et al. 2004). Pathogen movement in soil is generally affected by filtration and adsorption (Lewis et al. 1980). When studying the transport of multiple microorganisms (e.g. bacteria, protozoa, viruses) in soils the largest travel distance can be predicted for viruses (Hijnen et al. 2005). In case of viruses, removal appears to be dependent almost entirely on adsorption, which is a reversible phenomenon (Lewis et al. 1980). This adsorption can vary according to the strain and type of virus (Goyal and Gerba 1979; Landry et al. 1979). The most relevant factors controlling virus transport through soil, and hence influence adsorption, are soil type, water saturation state, pH, conductivity of the percolating water, and soluble organic matter (Bosch et al. 2006; Jin and Flury 2002).

INFLUENCE OF EXTREME WATER-RELATED WEATHER EVENTS

Extreme water-related weather events can influence the viral contamination of fresh produce. As such droughts or extended dry periods can reduce the volume of river flow and potentially increase the concentration of effluent-derived pathogens due to reduced dilution by stream-receiving waters (Cann et al. 2013). Heavy rainfall events can increase pathogen concentrations in receiving waters due to e.g. sewage overflow, bypass into local waterways and overwhelmed WWTP (Cann et al. 2013). These large precipitations can give rise to run-off water and flooding, which can spread fecal contamination to crops, both low growing and high growing crops (due to aerosols), receiving waters and agricultural farm land. Afterwards this contaminated soil can continue to be a reservoir for subsequent contamination through splash or direct contact with future fresh produce crop cycles. As such, NoV have been detected in urban floodwater in the Netherlands (de Man

et al. 2014) and the USA, but also in agricultural storm waters in Southeast of the USA (McBride et al. 2013). Next to surface waters, crops and agricultural farm land, also groundwater is particular vulnerable to contamination with enteric viruses during flooding conditions (saturated soil). As then adsorption of viruses to solids is reduced because virus contact with the soil has been diminished, and hence traveling time is much shorter (Bosch et al. 2006; Hunt et al. 2005). Hence the risk for contamination of the ground water increases.

1.2.1.4. Persistence on crops in the field

For persistence of enteric viruses in the environment, including fresh produce, temperature is the main influencing factor. Generally a faster die-off rate is observed on fresh produce than on or in soil, which is considered as a more protective environment from solar radiation and desiccation (Choi et al. 2004). However enteric viruses can persist for several days on fresh produce during pre-harvest conditions. As such a D-value of 4.8 days was observed for MNV-1 on semi-savoy spinach during a persistence study in greenhouse biocontrol chambers (Hirneisen and Kniel 2013c). While an inactivation rate (k_d) as low as 0.01, 0.12, and 0.11 per day (corresponding to a D-value of 100, 8, and 9 days) was observed for HAV on cantaloupe, lettuce and bell peppers respectively during a persistence study in a controlled environment chamber mimicking relevant growing condition in the USA and Central America (Stine et al. 2005a). All in all these limited studies suggest that enteric viruses persist longer than enteric bacteria and may persist from the time of contamination (e.g. by means of irrigation) to harvesting (Stine et al. 2005a). Persistence can depend on crop type and even crop variety (e.g. survival of MNV-1 and TV on semi-savoy spinach versus smooth spinach mentioned by Hirneisen and Kniel (2013c); Stine et al. 2005a; Carratala et al. 2013a). Since the surface texture and structure of vegetables may play an important role in the attachment and persistence of viruses. As such, the rougher or more irregular the surface of produce the longer viruses are able to persist (Stine et al. 2005a; Hirneisen and Kniel 2013c).

There has been evidence for biphasic inactivation of viruses on crops during pre-harvest conditions (Petterson et al. 2001b). An important implication of the biphasic inactivation is the possibility for virus accumulation on the crop surface over subsequent irrigations due to the presence of a persistent subpopulation of viruses that decay slowly (Petterson et al. 2001b). This higher persistence of this subpopulation could be a result of their location in a more protective niche such as stomata, complex wax structures, or cuts. Also the location of inoculum on the abaxial (lower) leaf surfaces has been observed to result in higher decimal reduction times (D-values) compared to viruses present on adaxial (upper) leaf surfaces (Hirneisen and Kniel 2013c).

1.2.1.5. Conclusion

In conclusion, proof of concept for different transmission route of enteric viruses at pre-harvest level is available in literature. However, these are mainly based on experimental studies as evidence derived from outbreaks is limited due to difficulties encountered during trace-back studies. Nevertheless proof of concept is strengthened by the occurrence of bacterial FBO and the occurrence and persistence of viral pathogens in soil (i.e. sludge (Prado et al. 2014)), irrigation water and on crops at pre-harvest stage (e.g. El-Senousy et al. 2013; Leon-Felix et al. 2010). High environmental persistence of enteric viral pathogens ensures the possible success of each of the transmission routes discussed. Hence good agricultural practices (GAP) and mitigation strategies related to the use of treated sludge and water in agricultural crop production, combined with appropriate precautionary actions and risk assessment in case of extreme weather events is requested.

1.2.2. Harvest and post-harvest contamination

In this stage food handlers are identified as critical point or hot spots for the transmission of foodborne viruses. Food handlers in this context include field harvesters, production plant workers, professional chefs and caterers, but also non-professionals such as those cooking at home, or e.g. at a youth camp preparing food. However, it could be that in contrast to institutional or private catering settings, viral foodborne infections in a home-setting are sometimes classified as a person-to-person infection instead of a foodborne infection in viral FBO statistics (Carter 2005). In the framework of this PhD thesis, the farm-to-fork chain will be restricted to the professional food handler or caterer at the retail stage.

The risk of contamination posed by an infected food handler can depend on personal factors specific to a food handler, including e.g. phase of clinical infection which impacts the degree of virus shedding, personal hygiene habits, and a variety of behavioral factors such as the willingness to work when feeling ill (Mokhtari and Jaykus 2009). Remark that this transfer by infected food handlers can involve both symptomatic as well as asymptomatic food handlers, as also asymptomatic food handlers can shed similar high loads of virus particles (Ozawa et al. 2007). Moreover, asymptomatic infections with NoV are quite common. For instance up to 14% of analyzed feces samples of asymptomatic food handlers working at a none-outbreak related facility in Japan tested positive for GII NoV (none were positive for GI NoV) (Okabayashi et al. 2008). Furthermore asymptomatic food handlers have been implicated in viral FBO (e.g. in Barrabeig et al. 2010; Daniels et al. 2000) and hence potentially play an important role in transmission of NoV. Remark that also mechanical transfer of viral particles from infected household members via the (asymptomatic) food handlers hands or clothing to the food has been

suggested as an important cause of outbreaks (Franck et al. 2015). In contrast in health care settings, asymptomatic health care workers, although shedding and hence contagious, are expected to have little, if any, role in transmitting the virus during outbreaks. This was most likely related to awareness and proper personal hygiene by the health care workers (Sukhrie et al. 2012).

In the next paragraphs the transmission during the harvesting phase and the processing phase will be discussed with special attention for the two case-studies soft red fruits (e.g. raspberries) and leafy greens (e.g. lettuce). Persistence onto the produce at the post-harvest stage and the effect of treatments used in the food processing of fresh produce on the viral load will be discussed in the next section (paragraph 1.3.).

1.2.2.1. Transmission during harvesting

The harvesting of fresh produce such as raspberries and lettuce can be either manual or mechanical. In case of **soft red fruits** such as raspberries, mechanical picker machines are available (Figure 1.7.). However depending on the region, scale and resources of the farm, still many fields are harvested manually. As such raspberries harvested in Serbia and Poland is still largely done by hand, while in the USA is mechanically harvested (95%) (Djurkovic 2012).

Figure 1.7. Mechanical harvesting of raspberries. (source: website Rader Farms: http://www.raderfarms.com)

Figure 1.8. Manual harvesting of crop lettuce.

In case of **leafy greens**, single leaf and multi leaf baby leafy greens are usually harvested mechanical, but lettuce crops such as butterhead lettuce or iceberg lettuce are mostly harvested by hand labor (Figure 1.8.). As such, contamination can take place due to **contaminated food handlers** and/or **contaminated surfaces**. These surfaces can get contaminated directly through contaminated workers, but contamination could also occur indirectly e.g. through the use of contaminated water for rinsing/washing or through cross-contamination from contaminated produce. Food handlers hands can also get contaminated by the produce and serve as a vehicle for further contamination. This was observed in a

study on hand hygiene of pickers of green bell peppers in Mexico where the workers' hands were not contaminated before work (0/36), while 13.9% (5/41) of the pickers' hands were contaminated with noroviruses after 3 h of work. In this study viral contaminated bell peppers were also collected directly from the field, hence contamination had to have occurred pre-harvest (Leon-Felix et al. 2010). During harvesting, food handlers such as fruit pickers have been suspected as the source of contamination in several reported viral soft red fruit outbreaks (e.g. Ramsay and Upton 1989; Reid and Robinson 1987 in FBO Table 1.1.).

To assess to which extent food handlers and contaminated food contact materials contribute to the introduction and spread of foodborne viruses, transfer experiments are available in literature that encompass all of the possible transfer combinations with hands – produce – food contact materials as either donor surface or acceptor surface (reviewed in Kotwal and Cannon 2014). In short, mean transfer rates of infectious viruses ranging from 2% to 18% and 0.1% to 2.3% have been found for contact of contaminated finger paths (dry conditions) with lettuce (Bidawid et al. 2004; Stals et al. 2013a) and berries (Verhaelen et al. 2013a), respectively. Identified variables that have a major influence on transfer rates are dry time of inoculum on donor surface (Sharps et al. 2012), moisture conditions of acceptor surface (D'Souza et al. 2006), and pressure and friction applied during transfer (Mbithi et al. 1992; Escudero et al. 2012).

These transfer rates might seem low, but keep in mind that NoV can be shed in concentrations up to 10^{12} genomic copies (RT-PCR) per gram (Atmar et al. 2008). Thus, as little as 0.0001 g of feces on a hand could contain up to 10^{8} of these viruses (worst-case situation) and hence a transfer of mere 0.1% could still result in a contamination up to 10^{5} viruses, indicating a potential food safety hazard. Enteric viruses have the potential to **persist on hands** for the better part of a work shift as in a study by Mbithi et al. (1992) a biphasic reduction curve was observed, resulting in a mere 0.5 to 0.8 $\log_{10}$ reduction four hours after inoculations of HAV on human hands. This illustrates the potential risk when infected food handlers are employed in a food processing/handling environment.

Once surfaces are contaminated, these surfaces can function as reservoir for further contamination events and this for prolonged periods of time, as the relative persistence of enteric viruses in the environment is high. As such the half-live of HAV on stainless steel under different conditions ($T \leq 20°C$ and $RH < 80\%$) was at least 4 days (Sattar et al. 2000). Surrogate MNV-1 has been observed to remain infectious after 28 days on several surfaces (stainless steel, ceramic, rubber, wood, glass, plastic) at room temperature (Kim et al. 2014). On inanimate surfaces, the most important factors that affect virus stability are the type of virus and surface, relative humidity, moisture content, temperature, composition of

the suspending medium, light exposure, and presence of antiviral chemicals or biological agents (Bosch et al. 2006). Next to these influencing factors, the presence of food residue has been observed to increase the persistence and the resistance of enteric viruses towards chemicals (Takahashi et al. 2011).

This type of transfer due to contaminated hands or surfaces **also** occurs during other stages **during post-harvest processing**, e.g. during sorting of the raspberries and during manual preparation of the produce at commercial/industrial settings or at home.

Generally, transfer due to contaminated hands is incited by a lack of hand hygiene, but mere hand washing alone will not remove all of the enteric organisms present (Mbithi et al. 1993; Todd et al. 2010). In a QMRA on the transfer of NoV to raspberries by infected food handlers it was shown that an intervention measure reducing the load of infectious virus particles on food handlers' hands, e.g. by hand washing, may lower the public health risk substantially (Verhaelen et al. 2013a). However, for hand washing to be successful in controlling viral foodborne disease outbreaks the presence of an effective disinfecting agent and adequate hand-washing instructions is not enough, as regular compliance must be enforced to make a significant difference (Papafragkou et al. 2006). It was also observed that for highly infectious pathogens as NoV, low transfer proportions could pose a greater public health risk as compared with high transfer proportions as a higher amount of produce (or acceptor surfaces) can get contaminated (Verhaelen et al. 2013a). For normal hand washing using non-antimicrobial soaps reductions between 0.7 and 1.8 $\log_{10}$ have been reported for removal of enteric viruses from hands (e.g. MNV-1 (Edmonds et al. 2012), HAV (Mbithi et al. 1993), RoV (Ansari et al. 1989)). However, among hand washing practices and sanitizers there is a large variability in effectiveness towards enteric viruses. This variability is due to the diversity in active compounds but also due to minimal differences in product formulations, differences in susceptibility of different (surrogate) enteric viruses used during testing and difference in the applied evaluation methodologies. As such, reductions up to 4 $\log_{10}$ of MNV-1 on finger pads have been observed for certain formulations based on alcohol (Steinmann et al. 2010; Belliot et al. 2008). While in other studies alcohol-based hand rubs (ABHR) turned to be relatively ineffective (Park et al. 2010a; Bidawid et al. 2004), as typical ABHR activity against non-enveloped enteric viruses varies depending on product formulation, e.g. the type, concentration of alcohol and other possible synergistic constituents. Overall, studies suggest that proper hand washing with soap and running water for at least 20 seconds is the most effective way to reduce norovirus contamination on the hands. However drying is also crucial since transfer ratios are considerably larger in case of wet donor or acceptor surfaces (D'Souza et al. 2006). Furthermore hand sanitizers might serve as an effective adjunct in between proper

hand washings but should not be considered a substitute for soap and water hand washing (Hall et al. 2011).

Contamination of lettuce could also occur at harvest level due to **cross-contamination** (by means of contact with contaminated crops) and **contaminated water** as in some cases lettuce is sprayed in the boxes with water just after harvesting to remove dirt, or used for cooling.

1.2.2.2. Transmission during post-harvest processing and handling

After harvest the produce is cooled at the farm or immediately after entering the post-harvest processing or distribution stage, depending on the locally available infrastructure. Both lettuce and soft red fruits such as raspberries can be sold fresh, without further processing, or moved to the post-harvest processing phase to be transformed into e.g. fresh-cut lettuce, individual quick frozen (IQF) raspberries or raspberry puree.

AT THE LEVEL OF AN INDUSTRIAL PROCESSING PLANT

In case of **raspberries** post-harvest **processing** can consist of the production of IQF raspberries or raspberry puree. For the production of IQF raspberries, raspberries are frozen after which manual sorting can take place (Figure 1.9.). The presence of NoV shedders is realistic considering the high prevalence of NoV infections in a community. Transmission through contaminated hands is hence realistic since the presence of NoV contamination on the hands of infected individuals has been confirmed during clinical trials (Liu et al. 2013). As such the presence of norovirus on hands of classifiers (53%) and packers (45%) prior to labor activities has been observed in a bell pepper packinghouse in Mexico (Leon-Felix et al. 2010). In case of the production of raspberry puree contamination by manual handling is not considered as a high risk but contaminated surfaces and cross-contamination by mixing of non-contaminated batches with a contaminated batch can occur. In conclusion, the main risk factors in the post-harvest processing of raspberries are contamination via contaminated hands (IQF raspberries) or surfaces (IQF and raspberry puree), which have been discussed in the previous paragraph.

Washing of raspberries is no normal procedure due to the damage washing causes to the fragile berries. However washing of other soft red fruits, such as strawberries, is commonly done before further processing. Since washing is one of the typical processing units in the production process of fresh-cut lettuce, risks concerning washing practices will be discussed for lettuce.

Figure 1.9. Manual sorting process of IQF raspberries (source: http://www.growingmagazine.com)

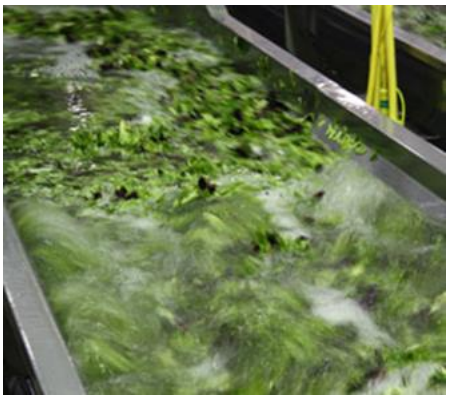


Figure 1.10. Industrial washing process of lettuce (source: http://www.scientificamerican.com).

In the **processing of lettuce** towards fresh-cut leafy greens, the produce is cut, washed (Figure 1.10) and spinned dry before packaging with or without protective atmosphere. This washing process has the potential to reduce the microbial load of the incoming fresh produce but has also the potential to be a direct source of contamination and a vehicle for spreading localized bacterial and viral contamination (cross-contamination) when sanitizers are used inadequately or are lacking. As such, in a recent outbreak in Korea the use of contaminated ground water during the processing of cabbage kimchi was identified as the source of viral contamination (Cho et al. 2014).Viral transfer from contaminated fresh produce to washing water (without sanitizers) has been documented for both lab-scale and industrial scale washing units for iceberg lettuce (Baert et al. 2009b) and strawberries (Casteel et al. 2009) respectively. However when adequate sanitizers were used no viruses were detected in the wash water in both studies. Transfer of viruses from the water phase to the lettuce has been observed in several studies as inoculation by immersion is a strategy used for inoculation (e.g. in Fino and Kniel 2008) in which the transfer of viruses from contaminated water to lettuce takes place instantly (Baert et al. 2008c). However in-depth studies on the consequence of a contaminated wash bath for e.g. the processing of several batches of lettuce and quantitative data of transfer rates is missing in literature. Persistence of enteric viruses in wash water has been shown to amply exceed common working hours (≥32h, 10°C) (Baert et al. 2009b) endorsing the potential risk for cross-contamination.

Next to the washing process, cross-contamination by contaminated machinery (e.g. cutters), contaminated surfaces, and leftovers from a previous contaminated batch is also a possibility. The possibility for such cross-contamination was experimentally demonstrated in a study tracking *E. coli* O157:H7-contaminated batch of leafy greens through a pilot-scale fresh-cut processing line (Buchholz et al. 2014).

AT THE LEVEL OF CATERERS AND PROFESSIONAL FOOD HANDLERS

At the level of **caterers and professional food handlers** the same risk factors exist as at the processing level. Consequently cross-contamination of lettuce can take place when several crops are washed using the same water. However the risk of cross-contamination will be somewhat lower due to the smaller scale of washing (washing of a few crops in catering versus washing batches up till 500 kg in the same water in industrial processing). On the other hand in this stage of the farm-to-fork chain the role of the **food handler** as cause of an outbreak is notorious as excessive handling of foods increases the opportunity of contamination. As such, infected food workers were implicated as the source in ca. 70% of reported FBO due to NoV in the USA (2009-2012) (Hall et al. 2014). Although bare-hand contact with food is likely the most prominent way of transmission (Hall et al. 2014), infected food handlers can also indirectly contaminate the food by contaminating the environment.

Examples of outbreaks due to indirect contamination routes, by means of contaminated surfaces, are at hand. For example, in an outbreak described by Patterson et al. (1997), over a hundred wedding guest got ill after eating norovirus (GII) contaminated potato salad. This contamination was due to environmental transmission since this potato salad was cleaned and prepared in a sink that was contaminated the evening before by a kitchen assistant that had vomited in this sink. Despite the fact the sink was cleaned with a chlorine based disinfectant, this sink was the source of the contamination as no other food prepared in the same kitchen was associated with the illness (Patterson et al. 1997). Nevertheless the use of sodium hypochlorite (chlorine bleach) is widely recommended to disinfect human NoV from surfaces. However when disinfecting (food contact) surfaces sufficient high concentrations of chlorine (if necessary with sufficient contact time) should be used *after* initial cleaning and scrubbing of the surfaces with water and soap (Tuladhar et al. 2012). Given that in lab experiments dried NoV surrogates on stainless steel surfaces were relative insensitive to 200 ppm chlorine concentrations (Cromeans et al. 2014). The CDC recommends in their report on NoV outbreak management and disease prevention guidelines the use of freshly prepared (use within 24 hours) concentrations of 1000 – 5000 ppm chlorine bleach solutions (Hall et al. 2011). However, these concentrations of NaOCl far exceeds what is mandated (i.e. 200 ppm determined as total available chlorine) for sanitizing food contact surfaces in the *Food Code* published by the US Food and Drug Administration (FDA) (21 CFR Part 178.1010). Furthermore an EPA-approved product list, although specified for use in health-care settings, has been published with commercial products besides sodium hypochlorite that are considered to be effective against NoV (http://www.epa.gov/oppad001/list_g_norovirus.pdf). For more information on surface disinfection related to NoV, relevant references to literature is provided (Bolton et al.

2013; Girard et al. 2010; Hoelzer et al. 2013; Tuladhar et al. 2012). Remark that cleaning cloths can play a role in spreading the viral contamination when not properly used (Gibson et al. 2012).

Despite the obligation for hand washing and cleaning, environmental contamination in catering companies is not uncommon. As such, in 13 of the 832 randomly chosen catering companies in the Netherlands that were not associated with recently reported outbreaks, swabs from kitchen surfaces were found positive for the presence of viral NoV RNA. However in 26 companies bathroom swabs turned positive for viral RNA, adding up to a presence of NoV RNA in 4.2% (35/832) of the randomly sampled catering companies (Boxman et al. 2011). Whether these 35 companies form an imminent threat for food safety is not derivable. But it is a fact that in the near past in each of these companies an infected food handler was present (or contact with an infected person occurred), lacking appropriate hand hygiene practices. As such, the risk for viral contamination of food was present.

Cross-contamination with naturally contaminated fresh produce or other food commodities such as seafood is also a risk factor. For example, the cross-contamination of salad by seafood was identified as the probable cause of an outbreak of NoV illness in 1979 (Griffin et al. 1982).

Kitchen utensils used during preparation of fresh produce can contribute to both removal and cross-contamination of microbial pathogens. Peeling of carrots and celery can for example result in a reduction in viral load (MNV-1 and HAV), although these reductions were rather modest (ca. 1 $\log_{10}$) with a high variability (standard deviation up till 1 $\log_{10}$). Peelers only partially removed viruses on the surface of carrots and celery, since cross-contamination of the peelers and transfer of virus to underlying tissues of the fresh produce likely occurs (Wang et al. 2013a). On the other hand, the utensils used during peeling and other practices such as cutting and grating can also give occasion for cross-contamination to initially uncontaminated produce. In this way, a contaminated peeler, knife or grater resulting from the preparation of a contaminated fresh produce item, could cross-contaminated seven, initially uncontaminated, successively prepared produce items (Wang et al. 2012; Wang et al. 2013a). However in these studies transfer was not quantified and the transfer experiment was stopped after 7 consecutive handlings. In a recent study by Shieh et al. (2014) the cross-contamination of a contaminated mechanical slicer, contaminated during slicing of a MNV-1 contaminated tomato, to subsequently initially non-contaminated tomatoes was quantified and modeled in order to serve as input data for risk assessment. According to this model it was found theoretical possible to cross-

contaminate over one thousand tomatoes with a single initial contaminated tomato containing 8 $\log_{10}$ RT-PCR units of virus (Shieh et al. 2014).

Exposure assessments that model the dynamics of the transmission of HuNoV in the retail food preparation environment are available (Mokhtari and Jaykus 2009; Stals et al., in press). In the model of Mokhtari and Jaykus (2009), gloving and hand-washing compliance were suggested as the most effective mitigation strategy for controlling contamination of a food product, when practiced simultaneously. The restroom environment has been identified as a major reservoir of NoV and hence an important contributor to contamination of foods (Mokhtari and Jaykus 2009; Verani et al. 2014).

1.3. EFFECT OF TREATMENTS USED IN FOOD PROCESSING OF FRESH PRODUCE ON FOODBORNE VIRUSES

In the previous section both known transmission routes and environmental persistence of enteric viruses such as NoV and HAV were discussed as both are key to identify possible prevention and control efforts for risk mitigation. However a third factor, i.e. the effect of treatments used in food processing of fresh produce on viruses, is also important to grasp the influence of the whole farm-to-fork chain on the viral load of fresh produce and for the identification of possible intervention strategies post-harvest.

In literature, review material on the effect of food processing techniques (Rivm 2013; Zuber et al. 2013; Hirneisen et al. 2010; Sanchez 2013) and preservation methods (Baert et al. 2009a) on the viral load of food products is abundant. Therefore this literature review will focus on treatments applicable to fresh produce that allow the retention of fresh-like organoleptic properties and the data will focus on effect of processing on the viral load of fresh produce. Special attention will be reserved for the two NoV – fresh produce commodities frequently linked to viral FBO, i.e. lettuce and raspberries. However, as *frozen* raspberries are frequently linked to viral outbreaks, leading to the recommendation to heat frozen berries before consumption in e.g. several Nordic countries, both freezing and heat treatment will also be included in this section.

1.3.1. Validation of effect of treatments – The necessity for surrogates

Treatments can result in inactivation of the present viral particles or the physical removal of viral particles. Removal refers to a distribution/partitioning process in which the virus particles are separated in a different fraction. Often this partitioning is not complete and can result in the presence of a subset of the originally present (infectious) viral units in the end product (Morgenthaler 2001). Examples of possible viral removal processes in food processing are peeling (discussed in §1.2.2.2.) and washing.

Inactivation is affected by some kind of physical or chemical change of the virus that results in the loss of the ability of the virus to infect host cells. This loss of infectivity can be either due to disruption of the capsid proteins, destruction of the viral RNA genome, or a combination of both previous modifications as schematically presented in Figure 1.4. For some inactivation treatments it is known what their primary mechanism of inactivation is, which can be either by damaging the viral coat (e.g. heat inactivation, high pressure processing), by impairing the genomic material, or by a combination of both (e.g. free chlorine) (Wigginton et al. 2012). After treatment, remnants of initially infectious virus particles (e.g. impaired virus particles or naked viral RNA) can still be present in the product and depending on which inactivation method has been used, can be detected by the

presence of e.g. immunologic epitopes or nucleic acids. As such, in order to measure the effect of inactivation the chosen assay must truly reflect virus infectivity and must hence be able to differentiate between infectious and non-infectious virus particles.

However due to the lack of a suitable cell-culture assay (CC) for infectious human NoV and other non-cultivable viruses, like most wild-type strains of HAV, the use of **surrogate viruses** is a necessity. Surrogate viruses are viruses related to the pathogens they have been chosen to represent (Richards 2012). A weakness inherent to the use of surrogates is that even though the surrogate can be closely related in genetic, physical, or chemical relatedness to the pathogen, differences between surrogates (Cromeans et al. 2014) and between surrogates and the viral pathogen (e.g. Leon et al. 2011) have been observed for different processing techniques. Experiments even show that different strains of a certain virus, e.g. different strains of cell culture-adapted HAV (Shimasaki et al. 2009) or FCV (Di Martino et al. 2010), can have different sensitivities to heat and high pressure. Also cell-culture adapted laboratory strains, as available for HAV, might not reflect the resistance of naturally occurring strains (Bertrand et al. 2012). This means that differences in resistance/susceptibility among different NoV strains (e.g. genotypes) should be anticipated (Richards 2012) as minor variations in structural or genomic components can have a marked impact on viral resistance to inactivation (Wigginton et al. 2012). As such, the use of surrogates is predicated on the assumption that they generally mimic the viruses they represent, although no direct correlation in the inactivation rates of the surrogates and the pathogens can be established. From a precautionary principle, the use of a 'worst-case' virus classified among the most persistent viruses has been suggested for the evaluation of inactivation treatments (Bertrand et al. 2012; Deboosere et al. 2010). This can be a different virus for different treatment and matrix combinations due to different sensitivity of the surrogates to certain inactivation treatments, hence justifying the use of multiple surrogate viruses when testing inactivation strategies. For instance, in complex matrices for inactivation at temperatures ≥50°C, the following ranking of heat sensitivity among frequently used (surrogate) viruses was observed: poliovirus > FCV > MNV > HAV > *Bacteroides fragilis* phage > Simian rotavirus > PhiX174 > F-specific RNA phages (e.g. MS2) (Bertrand et al. 2012). While in case of high pressure processing poliovirus is highly resistant compared to HAV and MNV-1 (Kingsley 2013; Kovac et al. 2012).

However, this could lead to an overestimation of the viral pathogens persistence and hence may over regulate the industry. On the other hand, the pathogenic virus could be more persistent than the surrogate viruses generally used during experiments (e.g. high pressure processing (Leon et al. 2011; Kingsley et al. 2007)), which would lead to insufficient food safety guidance. Surrogates most often used today are murine norovirus (MNV) and

Tulane virus (TV) (Kniel 2014) of which MNV is a member of the genus *Norovirus* classified in genogroup V and TV was only recently characterized as a representative of a new genus within *Caliciviridae*, the genus *Recovirus* (Farkas et al. 2008). However other surrogates such as Feline calicivirus (FCV) (genus *Vesivirus*) and bacteriophages such as MS2 continue to be used.

In order to obtain scientifically valid processing approaches to improve food safety, a shift to human volunteer studies has been suggested, although the latter does entail considerable costs (estimated to be ca. $500,000 (USA) (Richards 2012)). Still, viral loss of infectivity, either assessed by plaque assay, cell culture or feeding studies, is the most important and direct index for evaluating the effect of treatments concerning viral inactivation or removal. However, molecular methods, either in combination with an enzymatic pretreatment, cell/receptor binding assay or intercalating agents (e.g. propidium monoazide), are recently more and more used to evaluate the virucidal effectiveness of inactivation mechanisms (Knight et al. 2013). Possible strategies to differentiate between infective and non-infective viral particles were elaborately studied in a previous PhD study of this department (Li 2012) and hence will not be further discussed in detail. Despite the fact that it is now generally accepted that detection with classical molecular techniques may underestimate treatment efficiencies (e.g. heat treatment (Baert et al. 2008d; Bertrand et al. 2012) and high hydrostatic pressure (Kovac et al. 2012)), these new combination assays to differentiate infectious from inactivated viruses, have brought some new insights concerning viral inactivation and generally provide further evidence for the remarkable resistance of human NoV to several inactivation treatments (e.g. chemical sanitizers in Kingsley et al. 2014; HPP in Dancho et al. 2012; heat in Escudero-Abarca et al. 2014). However validation of these new assays using clinical trials should be undertaken in order to give assurance of their accuracy (Richards 2012). Therefore this review focuses on studies that examined the loss of infectivity and hence the inclusion of surrogate data was a necessity.

1.3.2. Effect of storage/preservation

Low temperature storage immediately upon harvest is recommended to preserve the quality of fresh produce, primarily by lowering the respiration and metabolism rates. Ideal storage temperatures for berries and leafy greens are 3-5°C and 0-5°C respectively (EFSA BIOHAZ Panel 2014b, 2014a). Temperature has been identified as the major factor influencing virus persistence. However in contrast to bacterial pathogens, maintaining the cold chain cannot be considered as a mitigation strategy for viral pathogens on fresh produce, as persistence of enteric viruses is higher at low temperatures, and decay rates generally increase with increasing temperatures (Rivm 2013). In Table 1.3., $\log_{10}$ reduction

data is presented for the persistence of enteric viruses or their surrogates on soft red fruits and leafy greens. Next to temperature, persistence has been found to depend on other factors such as the type of fresh produce (Croci et al. 2002; Verhaelen et al. 2012), different environmental factors (e.g. relative humidity (RH), presence of feces, aggregation) (Konowalchuk and Speirs 1975), and the virus type (Rzezutka and Cook 2004). As such the presence of fecal material strongly enhances virus persistence (Escudero et al. 2012). However the effect of RH is less unambiguous given that MNV and MS2 persisted better at low RH while HAV persisted better at higher RH in a study by Kim et al. (2012). However in a recent study it was suggested that absolute humidity (AH) rather than RH is the critical factor for keeping NoV infectious. The data also suggested that when the atmosphere was not entirely saturated (i.e., 100% RH), low AH values (below 0.007 kg water/kg air) are favorable to NoV persistence. This possibly explains the seasonality of NoV infections since low winter AH conditions (96.3% of the day with AH <0.007 kg water/ kg air) at temperate climates such as in Paris provides favorable conditions for keeping human NoV infectious (de la Noue et al. 2014).

Since the shelf-life of fresh produce, and especially for case-studies lettuce and raspberries, is short, only a low reduction in numbers of infectious viral particles is expected when stored at cold temperatures. Overall, persistence of enteric viruses can be expected during the time between purchase and consumption.

Table 1.3. Summary table of selected persistence studies on soft red fruits and leafy greens.

Matrix	Virus*	Storage condition	Log_{10} reduction (95% CI)	Reference
Strawberry	MNV	4°C , 7 days	0	Verhaelen et al. 2012
		10°C, 7 days	0.9 (0.7-1.0)	
		21°C, 3 days	1.4 (1.2-1.5)	
	FCV	4°C , 6 days	>1.5	Mattison et al. 2007
Raspberry	MNV	4°C , 7 days	0	Verhaelen et al. 2012
		10°C, 7 days	0.5 (0.3-0.6)	
		21°C, 3 days	1.1 (0.8-1.4)	
	PV	4°C , 9 days	0	Kurdziel et al. 2001
Lettuce	HAV	4°C , 7 days	2.0	Croci et al. 2002
	MNV-1	4°C , 11 days	Ca. 1	Escudero et al. 2012
	PV-1	4°C , 8 days	0.36	Yepiz-Gomez et al. 2013
	FCV	4°C , 7 days	Ca. 2	Mattison et al. 2007
		RT , 4 days	>2.7	
Spinach	HAV	5.4 ± 1.2°C, 14 days	1.0	Shieh et al. 2009

CI: confidence interval; '*': infectivity was assessed using cell-culture; RT: room temperature.

In case of fresh-cut lettuce, **modified atmosphere packaging** (MAP) is generally adopted. Next to functions such as the control of the respiration and reduction of enzymatic reactions such as browning, MAP conditions have also been designed to reduce the growth of spoilage microorganisms and pathogens. However, in a study on the persistence of HAV in packaged lettuce, a modified atmosphere did not influence the persistence when incubated at 4°C. Even a slight improvement in virus persistence on lettuce was observed in the presence of high CO_2 levels (70% CO_2, 43% persistence) at room temperature (RT) compared to when stored in bags with normal atmospheric conditions (6% persistence) (Bidawid et al. 2001). MAP is also applied on berries, however mainly on berries intended to ship fresh for long distances, and not applied in final consumer packages (EFSA BIOHAZ Panel 2014a).

Next to MAP packaging, also the antiviral activity of active packaging material consisting of silver-infused polylactide (PLA) films has been explored on virally contaminated vegetables. However, the efficiency of active packaging based on silver depends very much on the food type, on environmental factors, and on the pathogen. For instance on paprika no antiviral activity of the packaging towards FCV was observed, while reductions >3.5 $\log_{10}$ were observed for FCV on lettuce (Martinez-Abad et al. 2013).

The far most popular method for storing berries is **freezing**. As such in the two largest European raspberry producing countries Serbia and Poland, the majority of raspberries (>70% and >90% respectively), are exported frozen (Djurkovic 2012). Freezing however, has no pronounced influence on the viral load of fresh produce as no reduction was noted of MNV-1 surrogate on frozen onions and spinach after six months of storage (Baert et al. 2008c) and frozen storage for three months had limited effects on HAV and RV persistence in berries and herbs (Butot et al. 2008). Cryostability of NoV (GII.4) to freezing and thawing was also observed in a recent study by Richards et al. (2012). In general, freezing is actually used as a method for long-term storage of fecal and lysate stocks of enteric viruses in research. Also during outbreak investigations, according to the CDC's 'updated norovirus outbreak management and disease prevention guidelines', food samples strongly suspected as the source of an outbreak of acute gastroenteritis should be stored frozen at -20°C before analysis for optimal preservation (Hall et al. 2011). In conclusion, enteric viruses such as NoV and HAV are expected to persist during the shelf-life (up to 24 months and more) of frozen fruit and vegetable products and have been implicated in several FBO due to frozen berries (Table 1.1.).

Next to temperature, **pH** has been identified as a principal determinant for the growth of bacteria on fresh produce. Especially fruits can have an acidic internal pH far below the growth range of most bacteria (i.e. pH 4.4 – 9.8 (Devlieghere et al. 2011)). Berries have a

relative acidic internal pH varying between 2.7 and 4.5 (some blackberries), depending on the berry species (Knudsen et al. 2001). Raspberries have a pH varying between 2.9 and 3.5 (Bassett and McClure 2008). However, enteric viruses are engineered to persist the acidic stomach passage and hence long-term persistence (MNV-1, refrigeration temperatures) has been observed in acid conditions such as fruit juices (>21 days) (Horm and D'Souza 2011) and on berries (exceeding the shelf-life) (Verhaelen et al. 2012). MNV-1 has proved to be a suitable surrogate for human NoV in acid conditions (Cannon et al. 2006; Horm et al. 2012a; Seo et al. 2012). However, not all surrogate viruses of human NoV are unaffected by low pH. FCV has been observed to be more sensitive to low pH values and hence is considered to be a less appropriate surrogate for NoV in acid conditions (e.g. berries, fruit juices) (Butot et al. 2009; Cannon et al. 2006; Duizer et al. 2004; Horm and D'Souza 2011).

1.3.3. Effect of washing and sanitation

Besides the removal of dirt, foreign materials and tissue fluids from cut surfaces, washing, rinsing and spraying steps are the most commonly used processing steps to reduce the microbial load on fresh produce, while allowing the retention of fresh-like organoleptic properties. Often chemical sanitizers are added to the wash solution in order to maintain the water quality and to increase the reducing effect of the treatment. In this paragraph, efficiency of general washing practices and effect of chemical sanitizers will be discussed. However, comparing the outcome of different studies is not always relevant as several process parameters concerning the experimental set-up, such as treatment time and doses, produce:water ratio, organic load, pH of washing water and type of produce and virus, can have an influence on the effectiveness of decontamination treatments (Gil et al. 2009). An experimental set-up that mimics industrial practices as realistic as possible should be the intention.

1.3.3.1. Washing with water

Generally, washing results in ≤1 logarithm decrease (tenfold decrease) in the quantity of viruses detected (Li et al. 2011; Butot et al. 2008; Baert et al. 2008c; Dawson et al. 2005). Minor adaptations to the classic washing step by immersion such as usage of bubbling (Fraisse et al. 2011) or warm water (43°C) (Butot et al. 2008; Lukasik et al. 2003), or the inclusion of hand rubbing (Lukasik et al. 2003) did not significantly improve viral reduction on the produce. Household practices such as the addition of salt (2.0% NaCl), liquid dishwashing detergent (0.05%), or use of the consumer-oriented produce wash such as Fit (Proctor and Gamble) did not have any significant added value for reducing the viral load on strawberries in a study by Lukasik et al. (2003). In Table 1.4. a selection of

available reduction data is given when using tap water or the commonly used chlorine and peroxyacetic acid (PAA) solutions.

A major disadvantage of using just water during washing is the microbial build-up in the residual wash water (Baert et al. 2009b; Casteel et al. 2009). This can lead to cross-contamination between contaminated and initially non-contaminated crops, and will be further discussed in Chapter 5.

1.3.3.2. Washing with chlorine solutions

Chlorine is, despite its corrosive nature, the most commonly used sanitizing agent and widely applied in food processing. Chlorine is available as solid (calcium hypochlorite: $Ca(ClO)_2$), aqueous solution (sodium hypochlorite: NaOCl), and chlorine gas (Cl_2) (Van Haute 2014). Chlorine solutions can be either applied by immersion of the food crop or by spraying. After application of a sanitizer, in spray or in the form of a bath, rinsing or a final wash of the fresh produce in potable water is compulsory to remove any residual chemical and/or by-products (USA 21CFR173.315). In order to maximize the efficiency of chlorine disinfection, the concentration of free chlorine (FC), the pH (ideal pH 6 – 7) and the organic load (~COD level) of the wash water must be controlled.

Generally applied **chlorine** dosages and contact times by produce processors are 50 to 200 ppm (mg/L) for a maximal contact time of 1 to 2 min, leading to typical $\log_{10}$ reductions of 1 to 2 logs for bacteria and viruses on fresh produce (Goodburn and Wallace 2013; Casteel et al. 2008; Predmore and Li 2011). The effectiveness of chlorine in virus inactivation on produce can vary according to the virus under study (Fraisse et al. 2011; Butot et al. 2008) and according to the type of produce (Butot et al. 2008). In spite of the rather modest viral reductions on fresh produce obtained using chlorine, chlorine is much more effective for inactivation of viral pathogens in suspension, e.g. wash water, than for removal of these pathogens from fresh produce (Dawson et al. 2005). This reasoning also applies to bacteria. Hence, despite the general idea that sanitizers are used to reduce the microbial population on the produce, their main effect is maintaining the microbial quality of the water (Gil et al. 2009). Hence the use of wash water sanitizers is highly valuable to reduce cross-contamination from one contaminated crop/batch to the other crops/batches present in the washing bath.

Table 1.4. Subselection of available literature presenting the effectiveness of commonly used decontamination processes on the viral load of soft red fruits and leafy greens.

	Decontamination procedure (produce (g): water (ml) ratio)	Virus	Fresh produce	Log_{10} reduction	Reference
Tap water	0.5 min (15g : 200 ml)	HAV	Strawberry; raspberry Basil; parsley	0.8; 0.6 1.1; 0.5	Butot et al. 2008
	2 min (10g : 350 ml)	MNV-1	Spinach leaves	1.0	Baert et al. 2008c
	5 min (50g : 500 ml)	MNV-1	Lettuce	1.1	Baert et al. 2009b
	2 min, RT (50g : 2000 ml) 2 min, RT (50g : 4000 ml)	MNV-1	Strawberry; raspberry Cabbage; lettuce	0.8; 1.2 0.6; 0.2	Predmore and Li 2011
Chorine solutions	NaOCl 200 ppm, 0.5 min (15g : 200 ml)	HAV	Strawberry; raspberry Basil; parsley	1.8; 0.6 2.4; 1.4	Butot et al. 2008
	ClO_2 5 ppm, 10 min (15g : 200 ml)	HAV	Raspberries Parsley	1.0 1.1	
	NaOCl 200 ppm, 5 min (50g : 500 ml)	MNV-1	Lettuce	2.1	Baert et al. 2009b
	Chlorine 200 ppm, 2 min, RT, (50g : 2000 ml)	MNV-1	Strawberry; raspberry	1.0; 1.5	Predmore and Li 2011
	Chlorine 200 ppm, 2 min, RT, (50g : 4000 ml)	MNV-1	Cabbage, lettuce	1.3; 1.1	
PAA	80 ppm; 250 ppm, 5 min (50g : 500 ml)	MNV-1	Lettuce	1.9; 2.5	Baert et al. 2009b
	100 ppm, 2 min (25g : 500 ml)	MNV-1 HAV	Lettuce	2.4 0.7	Fraisse et al. 2011
	100 ppm, 2 min, 43°C (150g : 2000 ml)	MNV-1	Strawberry	1.8	Lukasik et al. 2003

PAA: peroxyacetic acid; RT: room temperature

The drawback for the use of chlorine is that this biocide is highly corrosive for the stainless steel surfaces frequently used in the food industry and its efficacy is negatively influenced by the organic load of the wash water. Also the formation of by-products in the wastewater, such as trihalomethanes (THMs), has been frequently cited as the downside of using chlorine and is the reason for the continuous search for new alternatives for disinfection (Fraisse et al. 2011). These by-products are formed by reaction of the chlorine disinfectant with organic matter in the wash bath. Despite the occurrence of the formation of THMs in the process wash water, no residue can be found in vegetable tissue after rinsing with tap water (Gomez-Lopez et al. 2013; Lopez-Galvez et al. 2010a). Hence, when good practices are applied (hence control of COD, FC and regular refreshing of washing water), chlorine-based sanitizers such as chlorine gas, sodium hypochlorite and calcium hypochlorite can be safely used to wash fresh produce, in spite of the formation of

THMs in the washing water. As such, suggestions that the industry should move away from this traditional disinfection agent are unfounded (Gil et al. 2009).

Another chlorine-containing disinfectant used in food production and processing is chlorine dioxide (ClO_2). Advantages of ClO_2 in comparison to the classic chlorine-containing disinfectants is that no formation of THM compounds occurs in the presence of organic matter (Lopez-Galvez et al. 2010a) and the sanitizer is little affected in its efficiency by pH and the presence of high amounts of organic matter (Hirneisen et al. 2010). However, application of ClO_2 in the USA is restricted for use in washing whole fruits and vegetables and hence not permitted for disinfection of fresh-cut fruits and vegetables (Hirneisen et al. 2010). Other restrictions for the use of chlorine dioxide are that it must be generated on site due to its instability and that it can be explosive when concentrated. Concentrated solutions of sodium chlorite are on the market, e.g. Carnebon 200 (International Dioxide Inc., Clark, N.J.) and Oxine (Bio-Cide International, Inc., Norman, Okla), that upon acidification generate "stabilized chlorine dioxide" (Lukasik et al. 2003). However, the effectiveness of ClO_2 at the recommended low concentrations for usage by the FDA (max. 5 mg/L or ppm), is rather low (ca. 1 $\log_{10}$ reduction) for FCV and HAV, even at the rather extensive contact times (10 min) tested by Butot et al. (2008).

1.3.3.3. Washing with other chemical agents

Next to chlorine containing solutions other chemical agents have been tested for their effectiveness in reducing the viral load of fresh produce during washing steps, e.g.: peroxyacetic acid solutions (PAA) (equilibrium mixture of hydrogen peroxide and acetic acid) (e.g. in Baert et al. 2009b; Allwood et al. 2004; Fraisse et al. 2011), the use of liquid or vaporized hydrogen peroxide (H_2O_2) (e.g. in Li et al. 2011; Lukasik et al. 2003), Ozone (O_3) (e.g. in Hirneisen et al. 2011; Hirneisen and Kniel 2013b), trisodium phosphate (TSP; Na_3PO_4) (e.g. in Lukasik et al. 2003; Su and D'Souza 2011), and surfactants (e.g. SDS in Predmore and Li 2011). Next to chemicals, also the antiviral properties of natural biochemical substances such as grape seed extract (GSE) in wash water (e.g. in Li et al. 2012; Su and D'Souza 2013) and sprays of essential oils (e.g. in Azizkhani et al. 2013) have been explored, although without success. Each of these tested substances have their own merits, limitations and drawbacks. Also the effect of some promising combinations of different chemicals or inactivation strategies have been studied on fresh produce. As such, the use of the combination of the surfactant SDS (50 ppm) and chlorine (200 ppm) enhanced the efficiency of virus removal and inactivation (MNV) resulting in a reduction mounting up to 3 $\log_{10}$ for lettuce, strawberries and raspberries (2 min, RT) (Predmore and Li 2011). Also a synergism was reported between the use of vaporized H_2O_2 and UV light on lettuce (Li et al. 2011; Xie et al. 2008).

In conclusion, good practices for washing of fresh produce require the use of a sanitizer. Sanitizers (e.g. Chlorine, PAA), however, are generally more effective in viral reduction in suspension (e.g. wash water) than on surfaces such as fresh produce (Dawson et al. 2005) as only marginal reductions of the viral load are obtained on fresh produce (1-2 $\log_{10}$). Even the introduction of multiple washing steps performed in series (Baert et al. 2008c), extended contact times (Xie et al. 2008), or the use of increasing concentration of disinfectants (Butot et al. 2008) will not necessarily lead to significantly higher reductions of the viral load of fresh produce. Similar to bacteria, viruses can be located in protective sites on the produce, such as the stomata or the cut edges, not accessible during washing procedures and most decontamination processes. As such, the use of sanitizers *in situ* during the wash process is primarily to maintain the microbial quality of the wash water and hence to limit the possibility of cross-contamination. Nonetheless, reporting of the effectiveness of the sanitizer under study in reducing the viral load in the resulting wash water is not always included (e.g. in Su and D'Souza 2011). However the latter is important to judge the utility for any sanitizers as mitigation strategy for cross-contamination. Good practices are also required to limit internalization of pathogens by avoiding influx of potentially contaminated wash water into the produce. Therefore a higher temperature of the washing solution than the temperature of the produce is demanded, as if the reverse is true, air bubbles inside the fresh produce will shrink upon contact with the cold water, resulting in a partial vacuum causing wash water to enter the tissue through pores, channels, or punctures (Holvoet 2014).

1.3.4. Effect of alternative strategies for decontamination

In this section the effect of radiation, both non-ionizing and ionizing radiation, and High Pressure Processing (HPP) will be discussed as non-thermal inactivation treatment options for enteric viruses in fresh produce. Both irradiation with ionizing radiation and appropriate use of HPP effectively inactivate both surface and internalized viruses.

1.3.4.1. Effect of radiation

Both ionizing and non-ionizing radiation have been tested as disinfection strategies for vegetables contaminated with viruses. The most widespread used form of **non-ionizing radiation** for decontamination is the use of *UV light* (100 – 400 nm). UV disinfection primarily occurs due to the germicidal action of UV-B (280 to 315 nm) and UV-C light (200 to 280 nm) on microorganisms (US EPA 2006). Most studies use low-pressure (LP) mercury lamps with a major wavelength output (85%) at 253.7 nm (monochromatic UV radiation) (Hijnen et al. 2006; Eischeid et al. 2011). Inactivation by this ultraviolet is based on the damage caused to the nucleic acids (DNA/RNA) of the cell or virus, of which the UV absorbance peaks near 260 nm. Among food- and waterborne pathogens, viruses are

generally more resistant than protozoa such as Cryptosporidium and Giardia, and the bacterial pathogens (Hijnen et al. 2006). Adenoviruses are the most UV-resistant class of viruses presently known and are therefore used as a standard for viral inactivation requirements in e.g. water disinfection (Eischeid et al. 2011). Concerning fresh produce, UV light (dose: 40-120 mW s/cm²) was shown to be effective in the reduction of HAV and FCV on lettuce and green onions, resulting in reduction of 4-5 $\log_{10}$ for HAV and 2.5-4 $\log_{10}$ for FCV. However on strawberries significantly lower reductions were observed for both viruses (i.e. <2 $\log_{10}$). In a study by Hirneisen and Kniel (2013b) however, MNV-1 proved to be more resistant to UV light, as a dose of 240 mW s/cm² resulted in a mere reduction of ca. 1.2 $\log_{10}$ on green onions. As such, the food matrix and surface typography play an obvious role (Fino and Kniel 2008). Also a wide variation in viral sensitivity to UV has been recognized (Eischeid et al. 2011), making it impossible to estimate the possible influence on human NoV.

Another disinfection strategy using non-ionizing radiation is the use of *pulsed light (PL) treatment*. PL is a modified and claimed improved version of delivering UV-C to bodies, using xenon lamps to deliver short time pulses of an intense broad spectrum (200 – 1100 nm) rich in UV-C light. PL treatment is a relatively new technology and only one study was identified applying PL treatment on produce. In this study the effectiveness of 10-30 pulses (300 µs each, fluence of 0.94 J cm^{-2} $pulse^{-1}$) was tested on MS2 inoculated on black pepper, chopped mint and garlic powder. However, only marginal reductions were obtained (generally <0.5 $\log_{10}$ reduction, except for mint 1.3 $\log_{10}$ reduction) in comparison to the reductions obtained in viral suspension after merely 4 pulses (i.e. >8 $\log_{10}$ reduction) (Belliot et al. 2013).

The downside of the use of non-ionizing radiation compared to ionizing radiation is the superficial character of UV treatment. The light should be able to reach all surfaces of the product and internalized microorganisms would be unaffected due to the light absorption by the surface. Therefore, this treatment option is quite impractical for decontamination of e.g. lettuce on an industrial scale.

Ionizing radiation is radiation that carries enough energy to liberate electrons from atoms or molecules, thereby ionizing them. Ionizing radiation used in food processing can be electromagnetic radiation (*gamma rays* and *X-rays*) or particulate radiation (*electron beam*). While the first type (γ-rays) are produced from a radioactive source (i.e. Co-60 or Cs-137), the other two (X-rays and e-beams) are produced by specific equipment converting other energy sources, such as electric current, without the involvement of any radioactive substance. As such, in the latter two cases the producing equipment can be switched on or off depending on the need (EFSA 2011). The application of X-rays will not

be further discussed as X-rays never found application in commercial food irradiation (RIVM 2013). Electron beam irradiation is a relatively new technology. In contrast to gamma rays and X-rays, electron beam's main disadvantage is the poor penetrative power. However for irradiation of e.g. pre-packed salads the penetration depth might be sufficient, if the produce is irradiated from two or more sites (Niemira 2003). Studies using electron beam (e.g. Espinosa et al. 2012; Sanglay et al. 2011) and γ-rays (e.g. Bidawid et al. 2000; Feng et al. 2011; Hsu et al. 2010) for decontamination of viral contaminated fresh produce are available in literature.

Viruses, having relatively little nuclear material and being small "targets", are relatively resistant to radiation compared to most vegetative bacteria (D_{10} values of 0.14 – 0.80 kGy) (EFSA 2011). Reported D_{10} values for enteric viruses/surrogates are e.g. 2.97 kGy for HAV on strawberries (γ-rays) (Bidawid et al. 2000) and 2.95 kGy for FeCV on lettuce (e-beam) (Zhou et al. 2011). However, D_{10} values are affected by a number of factors including temperature, water activity and chemical composition of the food (EFSA 2011).

Figure 1.11. International Radura logo, used to show a food has been treated with ionizing radiation.

For Europe, the Scientific Committee on Food (SCF) has expressed several opinions on irradiated foods and acceptable doses for specific food classes/commodities (e.g. in 1986, 1992, 1998). As such for vegetables and for fruits, overall average radiation doses (kGy) of up to 1 and up to 2 kGy respectively, were evaluated as acceptable. However, as regulated in the EU by Framework Directive 1999/2/EC and Implementing Directive 1999/3/EC, so far only "dried aromatic herbs, spices and vegetable seasoning" at the maximum overall absorbed radiation dose of 10 kGy is allowed (EFSA 2011). Currently, the US FDA approves doses up to 4 kGy to control foodborne pathogens in fresh iceberg lettuce and spinach (FDA 2008) as a response to three multistate outbreaks of *E. coli* O157:H7 traced to spinach and lettuce (CDC 2008). However, this irradiation dose proved impractical for the inactivation of viruses on fresh produce as only <2 $\log_{10}$ virus reduction of MNV-1 (4 kGy, γ-rays) was achieved on spinach, romaine lettuce, and strawberries in a study by Feng

et al. (2011) and a mere reduction of ≤0.70 $\log_{10}$ of MNV-1 was achieved on cabbage and strawberries in a study of Sanglay et al. (2011) (4 kGy, e-beam). As such, next to the inconvenience of a hesitant consumer acceptance towards irradiated food (logo: Figure 1.11.), the doses required for a meaningful reduction of viruses typically exceed legally approved doses and what most produce will tolerate in terms of changes in appearance, flavor, color, and texture (Fan et al. 2008).

1.3.4.2. Effect of high pressure processing

High pressure processing (HPP) is a non-thermal process that inactivates pathogenic and spoilage microorganisms as well as endogenous enzymes and has been used as a "cold pasteurization" method for fruit juices, fruit desserts, avocado-based products, sliced onions, and ready-to-eat vegetable dishes (Kingsley 2013; RIVM 2013). Pressures up to 1000 MPa are used that are instantaneously and uniformly transmitted throughout a sample, thus making this process independent on the shape or size of the food (Kingsley 2013; Kovac et al. 2010).

In research the effectiveness of HPP for viral inactivation has been tested on fresh produce matrices such as green onion slices (Kingsley et al. 2005), carrot juice, lettuce, blueberries (Li et al. 2013b), blueberry juice (Horm et al. 2012a), orange juice (Horm et al. 2012b), and different purees such as strawberry puree, lemon puree, tomato puree, watermelon puree and carrot puree (Lou et al. 2011). Although the resulting characteristics of the treated products are superior compared to heat-treated products, still HPP has been shown to affect sensory qualities such as color, texture, shape and rheological properties. However, these variable effects on the sensorial quality of fresh produce are depending on the pressure level and type of product (Kovac et al. 2010; Lou et al. 2011). As such, HPP has been recommended for processing of fruits intended for frozen storage, since freezing causes similar and more severe texture damage (Lou et al. 2011; Li et al. 2013b). Fresh produce related products such as purees, sauces, and juices are also fit for usage of HPP, as compared to intact fresh produce they lack the presence of intercellular air spaces that can be severely compressed during pressure treatment, inducing physical damage to the tissue (Li et al. 2013b).

Next to the treatment parameters such as pressure levels and treatment time, also the matrix can have a significant influence on the effectiveness of HPP (Kovac et al. 2012; Lou et al. 2011; Kingsley et al. 2005). Some parameters, such as temperature and pH (Li et al. 2013b; Lou et al. 2011; Kingsley and Chen 2009), were shown to influence the HPP inactivation of different types of viruses, in a contradictory way. For example, colder initial temperatures of the product enhanced the inactivation of human NoV GI.1 (Leon et al. 2011) and surrogates MNV-1, TV and FCV (Li et al. 2013b; Chen et al. 2005). In contrast,

HAV, a picornavirus, is more resistant to HPP at a lower temperature than at room temperature (Kingsley and Chen 2009). The same for the parameter pH, where human NoV (surrogates) tend to be more sensitive to HPP at neutral pH than at acidic pH (Lou et al. 2011; Li et al. 2013a). Whereas for HAV the opposite is true (Kingsley and Chen 2009). As such, direct validation of HPP conditions within the food or food matrix will be required, given the complexity of food matrices and the variable response of different viruses (Kingsley 2013).

Among enteric viruses a high variability in pressure resistance has been noted, even different virus strains can behave differently under pressure (Shimasaki et al. 2009). As such, it is conceivable that different human NoV genogroups, and perhaps different clusters within a human NoV genogroup, would exhibit varied sensitivities to HPP (Leon et al. 2011). During testing, human NoV surrogates FCV and TV proved to be more susceptible to HPP compared to MNV-1 (Li et al. 2013b; Horm et al. 2012a). In a study by Lou et al. (2011) for application of HPP in the fresh produce industry, the optimal condition for MNV-1 inactivation by HPP in diverse fresh produce matrices was determined to be refrigeration temperature with a treatment pressure of 450 MPa and a holding time of 2 min. Using these conditions viral reductions between 4.7 and 7.0 $\log_{10}$ were obtained without significantly altering the physical quality of the food samples (Lou et al. 2011). However, when the inactivation kinetics of MNV-1 (cell-culture) are compared to human NoV GI.1 (obtained during a human feeding study using infected high pressure processed oysters), human NoV might be more resistant to HPP than MNV-1. As in the human feeding study of Leon et al. (2011) treatment of oyster (seeded by injection) by HPP at 400 MPa, for 5 min at 6°C was insufficient to prevent NoV infection in human volunteers, suggesting that 4 $\log_{10}$ genome equivalent reduction was not achieved. While a 5 min, 400 MPa treatment at 5°C was sufficient to inactivate 4.1 $\log_{10}$ PFU MNV-1 in oyster tissue (Kingsley et al. 2007). In the human volunteer study a 600 MPa treatment for 5 min at 6°C was successful to inactivate human NoV GI.1 within raw oysters (Leon et al. 2011). This higher resistance of human NoV to HPP was also observed when binding assays using porcine gastric mucin-conjugated magnetic beads followed by RT-qPCR assays were used for discriminating potentially infectious human noroviruses GI.1 (Dancho et al. 2012) and GII.4 (Li et al. 2013a) following HPP.

1.3.5. Effect of thermal treatment

In food processing thermal processing is a classic inactivation strategy which involves heating of a food product at a temperature that ranges from 50 to 150°C, primary to inactivate foodborne pathogens and to inactivate endogenous enzymes. In light of the main identified food commodities of concern, namely soft red fruits and leafy greens, this

paragraph will be restricted to the effect of classic heat treatment (e.g. pasteurization) and blanching, since heating of frozen berries has been regularly communicated as a mitigation strategy for FBO due to viral contaminated berries (Figure 1.12.) (Guzman-Herrador et al. 2014). The possible effectiveness of this measure was illustrated during the German outbreak in 2012 due to contaminated frozen strawberries. During outbreak investigation it was noted that not all kitchens which used the implicated batch of frozen strawberries were linked to disease cases. This was a result of the different ways of preparing the strawberry compote among kitchens. As such, it was observed that those places receiving meals from kitchens where the strawberry compote was stewed (thoroughly cooked), were not affected by outbreaks. The schools and childcare facilities that received the compote from kitchens that did not sufficiently heat the compote were indeed obviously associated with disease cases (Task Force gastroenteritis 2012).

Figure 1.12. Label of frozen raspberries (Hallon in Swedish) stating that the raspberries should be cooked at 90°C for at least 2 min before eating.

In literature, an overall lower virus sensitivity to temperature change has been noted in complex matrices (e.g. dairy and food products) compared to simple matrices (e.g. drinking water and synthetic media) at the high temperature range (>50°C) (Bertrand et al. 2012). Hence, validation of a specific heat treatment in the relevant food matrix is well-considered. However in literature only a limited number of heat inactivation studies are available for produce matrices. Relevant matrices used for traditional heat inactivation experiments of enteric viruses/surrogates are restricted to purees of soft red fruits (e.g. raspberry, strawberry, bilberry) (Baert et al. 2008b; Deboosere et al. 2004; Deboosere et al. 2010) and spinach (Bozkurt et al. 2014a).

The risk of NoV infection remains associated with mildly pasteurized (30s at 65°C and 15s at 75°C) raspberry puree, since reductions less than 3 $\log_{10}$ were obtained for MNV-1 (Baert et al. 2008b). The inadequacy of mild heat treatment steps at low temperatures can also be confirmed by a human challenge study in which human norovirus was found to remain infectious for volunteers after 30 min at 60°C (Dolin et al. 1972). Next to the virus type, matrix factors such as pH and sugar content have been confirmed to have a significant effect on heat inactivation of enteric viruses and surrogates. As such, studies are available that observed a rise in inactivation time of HAV (in strawberry mashes) and MNV-1 (in PBS), with increasing sucrose concentration and observed moderate rise in inactivation time of HAV (in strawberry mashes) with increasing pH. Attempts have been made to model the heat inactivation of HAV in berry mashes in function of temperature and product characteristics such as pH and sugar concentration (e.g. Deboosere et al. (2004) using synthetic media to mimic chemical characteristics of strawberry mashes and Deboosere et al. (2010) on berry mashes). However validation of the model by Deboosere et al. (2004) in fruit-based products failed. Weaknesses of the latter model of Deboosere et al. (2010) are the limited temperature range (i.e. 65 to 75°C) and the inclusion of the come-up time (approx. 2 min) in the treatment time. This practice assumes that the temperature during this come-up time was constant and at the target temperature, and possibly explains the occurrence of a shoulder in the inactivation curves and the very low log reduction estimates when the model was used to calculate the effect of short heat treatments (e.g. 0.02 and 0.16 $\log_{10}$ reduction at 30s and 1 min at 75°C and pH 2.5 respectively). In contrast, in-house data on MNV-1 heat inactivation in raspberry puree (75°C, 30 s) suggests a reduction of $\geq$4.29 $\log_{10}$ (non-published data). As such, there is a need for additional studies that take into account heat-inactivation kinetics during the phase of temperature increase to reach the target temperature (Deboosere et al. 2010) or models that do not include the preheating step at all. As in validation of time/temperature treatments in food processing, generally preheating and longer exposure to these temperatures during cooling down are not included to assume a worst case scenario in which the reduction solely originates from the actual heat treatment (Baert et al. 2008b).

Another relevant thermal treatment process is blanching. Blanching is a thermal pretreatment (between 75°C and 105°C) that is generally conducted prior to freezing and canning to inactivate micro-organisms and enzymes and remove entrapped air. Both the hot water bath blanching process and steam blanching have been proven to be effective. As such, a reduction of $\geq$2.44 log MNV-1 (detection limit of assay was reached) was observed when fresh spinach was treated 1 min in a hot water bath of either 80°C or 90°C (Baert et al. 2008c). Confirmation of the effectiveness of (steam) blanching was provided in a study on fresh herbs such as parsley, basil, mint, and chives. Generally >3 $\log_{10}$ reductions were

observed for HAV and FCV on fresh herbs when blanched at 95°C for 2.5 min. When blanching was performed at 75°C for 2.5 min, more variation in heat resistance of enteric viruses was observed, varying depending on the herb (e.g. HAV reduction on mint and chives was 1.7 and >3.0 $\log_{10}$ respectively) (Butot et al. 2009).

In general, heat inactivation studies indicate that mild thermal inactivation methods (such as pasteurization) may not be stringent enough to eliminate human NoV (Escudero-Abarca et al. 2014; Baert et al. 2008b). However, cooking procedures in which an internal temperature of the food reaches at least *90°C for 90 seconds* are considered adequate treatments to destroy viral infectivity in most foods (Anonymous 2012d; FAO/WHO 2012). Following the recommendation for heat treatment of shellfish (90°C for 90 s), HAV was successfully inactivated in shellfish (Hewitt and Greening 2006). Several other thermal studies suggest that high temperature, short time treatments (e.g. 90°C, 30 s) should suffice for inactivation (>4 $\log_{10}$ reduction) of human NoV (surrogates) (e.g. Bozkurt et al. 2014b, in-house data on MNV-1 reduction in raspberry puree: ≥4 $\log_{10}$ for 95°C, 30 s). Nevertheless, data concerning heat treatments of produce at temperatures >75°C are scarce and only available for a limited number of surrogates (e.g. Deboosere et al. 2004 for HAV). Hence additional relevant heat inactivation studies for this high temperature range in relevant produce matrices and for several (surrogate) viruses should be conducted to obtain more insight.

1.3.6. Conclusions

Overall, human NoV are introduced in the fresh produce chain by human fecal pollution and food handlers are believed to play a significant role. The high persistence of NoV in the environment combined with high resistance of NoV to commonly used decontamination practices (e.g. washing) of fresh produce, ensures the persistence of NoV between contamination and consumption due to the relative short shelf life of fresh produce. As such, effective control strategies need to focus on prevention of contamination and to limit cross-contamination. The most important routes identified in this literature study are contaminated food handlers, justifying the need for creating awareness on the issue of NoV and HAV and education of food handlers in good hygienic practices (GHP). In addition, contaminated irrigation water and process water have been shown to be relevant viral contamination routes of fresh produce. This introduces the need for assessment of the risk associated with the irrigation water source used, the implementation of proper water treatment options, and in case of washing of fresh produce the inclusion of good practices, including the correct use of sanitizers *in situ* the wash process.

CHAPTER 2

EVALUATION OF VIRAL CONCENTRATION METHODS FROM IRRIGATION AND PROCESSING WATER

Redrafted from

De Keuckelaere, A., Baert, L. Duarte, A., Stals, A., Uyttendaele, M. (2013). Evaluation of viral concentration methods from irrigation and processing water. Journal of Virological Methods 187, 294-303.

and De Keuckelaere, A., Stals, A., Baert, L., Uyttendaele, M. (2013). Performance of two real-time RT-PCR assays for the quantification of GI and GII noroviruses and hepatitis A virus in environmental water samples. Food Analytical Methods 6, 1016-1023.

Experimental set-up for viral concentration from water using the VIRADEL principle.

Authors contributions

Conception, planning, interpretation, and writing of the paper was done by Ann De Keuckelaere, Leen Baert, and Mieke Uyttendaele. Interpretation of the results and major contributions on writing style were also made by Ambroos Stals. The practical lab work was performed by Ann De Keuckelaere and Alexandra Duarte.

2. EVALUATION OF VIRAL CONCENTRATION METHODS FROM IRRIGATION AND PROCESSING WATER

2.1. *ABSTRACT*

Four viral concentration methods were evaluated for their efficiency in recovering murine norovirus-1 (MNV-1) (surrogate for human noroviruses (NoV)) and MS2 bacteriophages from processing water (1 L) and four different types of irrigation water (bore hole water, rain water, open well and river water) (2-5 L). Three methods were based on the viral adsorption and elution principle, two methods using an electronegative HA-membrane (Katayama et al., 2002), one method used an electropositive Zetapor membrane according to CEN/TC275/WG6/TAG4 and the fourth method was based on size exclusion using a tangential flow filtration system. Detection of MNV-1 was achieved by real-time reverse transcription polymerase chain reaction (RT-qPCR) and detection of MS2 by double-layer plaque assay. For the recovery of MNV-1, the method using an electronegative HA-filter in combination with an elution buffer earlier optimized by Hamza et al. (2009) (Method 1) performed best for all types of water (recovery: 5.8 - 21.9%). In case of MS2 detection, the best method depended upon the type of water although Method 1 provided the most consistent recovery.

To complete this evaluation, Method 1 was evaluated further for the concentration of human enteric viruses (GI and GII NoV, hepatitis A virus (HAV) and rotaviruses) in the same five types of water. At the same time the performance of two RT-qPCR assays, an in-house two-step RT-qPCR versus a commercial one-step RT-qPCR assay, was tested for the detection of HAV and GI and GII NoV in these environmental water samples. Regarding the performance of the two RT-qPCR, only minor differences were observed, however the commercial one-step RT-qPCR seemed somewhat better optimized for environmental samples since slightly less inhibition was noticed in the undiluted RNA samples compared to the in-house assays. Hence the results using the commercial assay were further used for assessment of detection of human enteric viruses. Method 1 proved reliable for the detection of NoV and HAV in all water types, although detection of rotaviruses (RV) was somewhat less efficient. Mean recovery efficiencies ranged from 4.8% for detection of GI NoV in open well water to 32.1% for detection of HAV in bore hole water, depending on the water type and the viral pathogen analyzed.

2.2. INTRODUCTION

Viral pathogens, such as human infectious NoV and HAV, play a significant role in foodborne outbreaks throughout Europe and the USA (CDC 2009; EFSA 2010). Fresh produce are, next to shellfish and ready-to-eat foods, recognized as an important vehicle in the transmission of foodborne viral outbreaks (FAO/WHO 2008b).

Fresh produce can be contaminated at the pre-harvest stage by contact with viral contaminated water or sludge or both at the pre-harvest and post-harvest stage by contact with asymptomatic or symptomatic infected food handlers, contaminated processing water or surfaces (Baert et al. 2009b; Carter 2005; Leon-Felix et al. 2010; Seymour and Appleton 2001). Whereas human activity and thus food handler's contamination is an established source of foodborne viruses and can be controlled by good hygienic practices and training, little knowledge is available on the prevalence of foodborne viruses in irrigation water or water used in post-harvest processes such as washing and rinsing, and thus its role for acting as a vehicle of transmission to fresh produce crops. Several sources of water are applied for irrigation of crops and this may range from ground water and collected rain fall (general assumed to be of good and even potable water quality) to surface water (streams, rivers) and may include in some regions also insufficiently treated wastewater with variable microbial quality (Pachepsky et al. 2011). Microbial quality is generally measured by the use of bacterial indicator organisms such as coliforms, fecal coliforms and *Escherichia coli* but these indicator organisms may not be an accurate reflection of enteric virus presence (Jurzik et al. 2010; Steele and Odumeru 2004). The results of previous screenings in water have shown that human enteric viruses are abundantly present in diverse ranges of water sources in the environment worldwide (Lodder et al. 2010; Miagostovich et al. 2008; Victoria et al. 2010; Wyn-Jones et al. 2011). River water samples are likely to be contaminated as they are fed continuously with effluents of waste water treatment plants, which are optimized for the removal of bacteria and are less effective in removing viruses (da Silva et al. 2007; Hewitt et al. 2011; Maunula et al. 2012; Ueki et al. 2005). Even though river water is one of the irrigation water sources that is most likely to be contaminated with hazardous microorganisms, this water type is in most parts of the world most commonly used for irrigation of salads which are to be consumed raw (Knox et al. 2011). Even in water sources considered relatively safe for bacterial contamination, such as ground water, viruses could be detected (Cheong et al. 2009; Park et al. 2010; Steyer et al. 2011). Although a link has been made between the detection of human enteric viruses on the irrigated vegetables and in the irrigation water (Cheong et al. 2009; van Zyl et al. 2006), at present few studies have looked into the prevalence of foodborne viruses such as NoV, HAV and RV in sources of irrigation water in order to estimate the possibility of viral contamination of fresh produce irrigated with these waters.

This in part due to the need of laborious viral concentration and detection methods and the insufficient knowledge on their performance in these types of water matrices. As such, most studies that concentrate on the performance of different viral concentration methods from water, focus on other types of water such as standardized water (e.g. distilled water) (Lee et al. 2011), drinking water, source water for drinking water production (Gibson and Schwab 2011), influent and effluent waters in order to evaluate the efficiency of waste water treatment plants in their removal of viruses (Albinana-Gimenez et al. 2009; Wyn-Jones et al. 2000), and seawater as production area of bivalve mollusks or recreational zone (Gibbons et al. 2010).

The aim of this study was to select an appropriate viral concentration method for monitoring the presence of foodborne viruses in different types of irrigation water and also post-harvest washing water used in fresh-cut produce industry. To our knowledge this is the first study that attempts to evaluate different viral concentration methods for the detection of foodborne viruses in various types of water applied commonly in the horticultural sector during agricultural production and further processing. In the current study two different approaches to concentrate viruses in water were evaluated. Three methods applying the VIrus ADsorption and ELution (VIRADEL) principle, based on a protocol described by CEN/TC275/WG6/TAG4, Hamza et al. (2009), Katayama et al. (2002) and Wyn-Jones et al. (2011), were compared to a single method based on the principle of size-exclusion, more specifically ultra-filtration using a tangential flow filtration system previously validated by Ceeram (La Chapelle-sur-Erdre, France). MNV-1 and bacteriophage MS2 served as human enteric viral pathogen surrogates for the evaluation of the four viral concentration methods, in four types of irrigation water (bore hole water, rain water, open well water, river water) and one type of processing water. A wide range of irrigation water types were chosen as it is clear from previous experiments that a single viral concentration method can show different recovery efficiencies depending on the type of water examined (Haramoto et al. 2009; Lewis et al. 2000;Victoria et al. 2009). For the detection of MNV-1, molecular methods were selected for detection since for the detection of major food-/waterborne enteric viruses an appropriate cell culture does not exist (Koopmans and Duizer 2004).

Based on the comparison of the different viral concentration methods, one method was selected for further evaluation with a broad panel of human enteric viruses, including GI and GII NoV, RV and HAV, in the same five types of water. Furthermore, for the detection of GI, GII NoV and HAV the performance of commercially available one-step RT-qPCR detection kits for the separate detection of GI, GII NoV and HAV (Ceeram, La Chapelle-sur-Erdre, France) was compared with an in-house two-step duplex RT-qPCR method for

the detection of GI and GII NoV (Stals et al. 2009a) and with an in-house adapted two-step RT-qPCR protocol derived from literature (Costafreda et al. 2006) for the detection of HAV. The goal was to assess both assays in their performance for the detection of NoV and HAV in the presence of possible inhibitors naturally present in environmental water samples.

2.3. MATERIALS AND METHODS

2.3.1. Water samples

Four primary concentration methods were tested for their efficiency in recovering viruses from various types of water. For the purpose of this study all types of irrigation water samples – bore hole water, rain water, open well water and river water - were collected in an agricultural area surrounding the city of Ghent (Belgium). Bore hole water was taken from a 9 m deep well in sandy soil. Rain water was collected from an underground tank where the water was stored after it was collected initially from a roof top situated in an agricultural area. Open well water was collected from an open well situated in a pasture. River water was taken from the 'Oude Leie' (an old cut off part of the river 'Leie') before entering Ghent, while processing water was taken from a local fresh cut lettuce processing plant from the water bath at the end of the washing stage of mixed salad. The processing water was dechlorinated by the addition of 100 mg/L sodium thiosulfate (Sigma-Aldrich, Steinheim, Germany). Water samples of each source were taken on two different occasions: during spring and at the end of the summer. Sampling volumes of 1 - 5 L were processed when possible (depending on the method and type of water sample). Water quality parameters analyzed just before filtration were pH and total suspended solids (TSS) according to Standard Methods (1998) (APHA 1998). Samples were stored at 4°C for a maximum of 48 hours before use in the experimental set-up.

2.3.2. Artificial contamination of water samples

For the initial comparison of the four different viral concentration methods, water samples were contaminated artificially with MNV-1, a human NoV surrogate kindly provided by Prof. H. W. Virgin, and with MS2 bacteriophages, kindly provided by the Flemish Institute of Biotechnology (VIB, Ghent, Belgium). MNV-1 was cultured as described earlier (Wobus et al., 2004) and MS2 was cultured according to ISO 10705-1 (ISO 1995). Stock dilutions of MNV-1 and MS2 were prepared in respectively PBS (Lonza, Verviers, Belgium) and PPS and stored in aliquots at -80°C until use. Concentrations of MNV-1 genomic copies and MS2 titer (in PFU) were respectively determined by real-time RT-PCR (Baert et al. 2008d; Stals et al. 2009a) and double-layer plaque assay, ISO 10705-1

(ISO 1995). Water samples were spiked to a final concentration of approx. 7 log MNV-1 genomic copies/L and approx. 7 log MS2 PFU/L.

For the further evaluation of the selected viral concentration method, the five different types of water were contaminated artificially with NoV, RV and HAV. NoV GI.4 and GII.4, and rotavirus G1P[8] stool samples were kindly provided by the National Institute for Public Health and the Environment (RIVM, Bilthoven, the Netherlands). From these samples a 10% stool suspension was made in PBS (Lonza) and subjected to centrifugation (2000 ×g, 15 min, room temperature (RT)). The supernatant was transferred to a new tube. From this solution tenfold dilutions were made in PBS (Lonza). HAV lysate (HM-175) was cultured in FRhK-4 cells as previously described (Nasser and Metcalf 1987), although slightly modified. HM-175 quantification was performed by $TCID_{50}$ and an agar overlay plaque assay. Dilutions of this HAV lysate were made in PBS (Lonza). Aliquots of all the spikes were stored at -80°C until use. Concentrations of genomic copies of all human enteric virus inoculums were determined by real-time RT-PCR. Water samples were spiked to obtain concentration of approx. 6 log GI.4 NoV genomic copies/L, 7 log GII.4 NoV genomic copies/L, 7 log HAV genomic copies/L (estimated final titer of approx. 10^6 $TCID_{50}$/ L water sample) and approx. 7 log RV RT-PCRU/L.

2.3.3. Virus concentration methods

Four viral concentration methods were tested for their efficiency in recovering MNV-1 and MS2 phages in four types of irrigation water (bore hole water, rain water, open well water and river water) and in fresh cut lettuce processing water, all inoculated as stated above. An overview of the viral concentration methods evaluated in this study is presented in Figure 2.1. Methods 1, 2 and 3 are based on the virus adsorption and elution (VIRADEL) principle and used an electronegative (HA-filter, Millipore) or an electropositive filter (Zetapor, 3M) (Wyn-Jones and Sellwood 2001). As viruses are normally negatively charged in the environment, viruses can adsorb to the electropositive membrane by electrostatic interactions. In case an electronegative membrane is used, the water samples need to be preconditioned by lowering the pH of the water sample below the virus isoelectric point and by addition of magnesium chloride (Ikner et al. 2012) in order to adsorb the viruses by electrostatic and hydrophobic interactions (Lukasik et al. 2000; Shields and Farrah 1983). In these methods, membranes with a diameter of 142 mm were used in a pressure pump system for water filtration (Millipore, Bedford, MA, USA). Maximum filtration speed was set at 30 L/h. In the fourth method, based on size exclusion, a tangential flow filtration system was used (Vivaflow 50 cassettes of Sartorius (Goettingen, Germany), molecular weight cut-off value (MWCO) = 50 000). For the various types of irrigation water, bore hole water, rain water, open well water and river

water, a sample volume of 5 L was used for Methods 1, 2 and 3 and 2 L for Method 4. In case of the processing water, 1 L was used in all methods. As this study determined the performance of different viral concentration methods in subsamples of the same water sample, variation due to differences in water chemistry were minimized. Additionally, the indigenous contamination of GI and GII NoV in all water types was examined using the four viral concentration methods in combination with a recently developed in-house two-step RT-qPCR (Stals et al. 2009a).

Subsequently, Method 1 was evaluated further concerning its efficiency in recovering human enteric viruses like NoV (GI.4 and GII.4), HAV and RV. All sample volumes were the same with the exception of the open well water where only 2 L could be processed due to a higher presence of algae blocking the filter. All viral concentration and extraction experiments, for each method and each type of water, were done in triplicate.

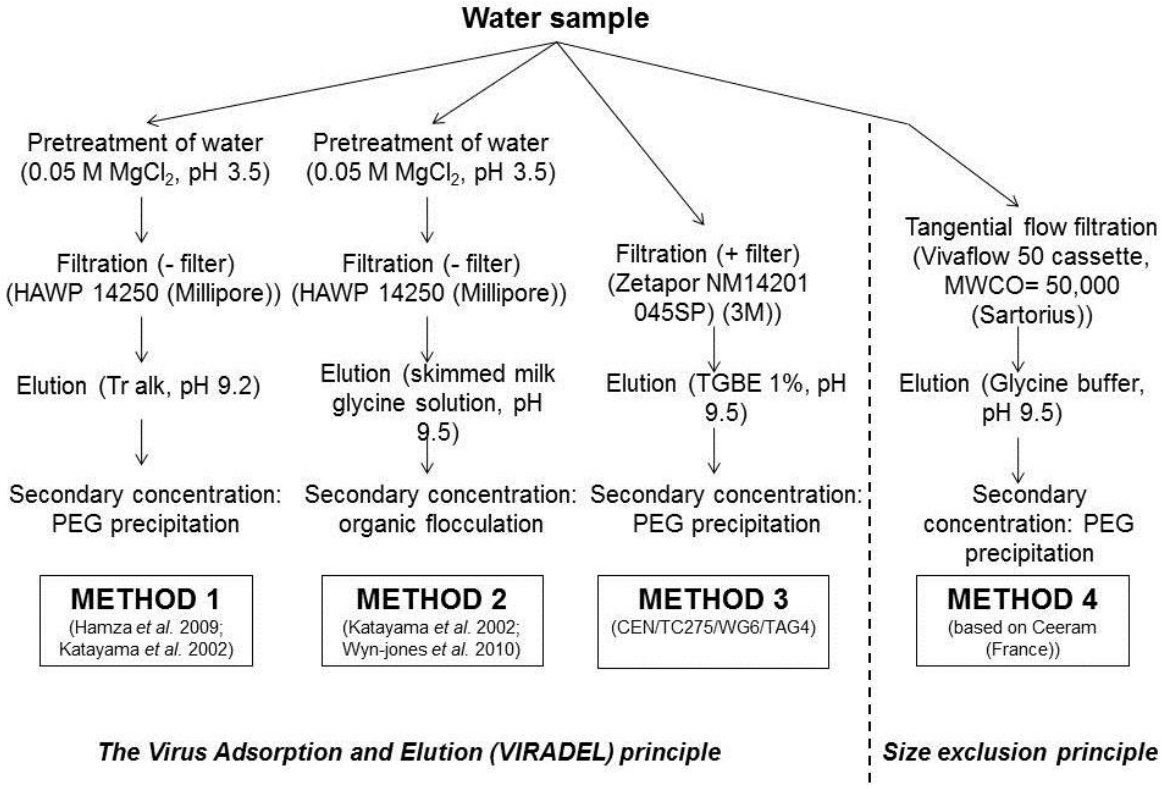


Figure 2.5. Schematic overview of the different viral concentration methods evaluated for the recovery of MNV-1 and MS2 phages

2.3.3.1. Method 1

Method 1 (HA-Tr alk) was elaborated based upon a previously reported method by Katayama et al. (2002) with an elution buffer optimized by Hamza et al. (2009). Briefly, a water sample volume of 1 - 5 L, depending on the water type, was spiked with viruses and $MgCl_2$ was added to a final concentration of 0.05 M. Finally the pH was adjusted to 3.5

with 1 M HCl. A type HA negatively charged membrane (HAWP14250, Millipore, Ireland) with a 0.45 µm pore size and a 142 mm diameter was used in a pressure pump system for water filtration. Glass fiber filters (AP15 and AP20, Millipore, Ireland) were used as prefilters to delay clogging of the HA-filter. After filtration the membranes were rinsed with 300 ml 0.5 mM H_2SO_4 (pH 3.4) to facilitate the elution with 70 ml Tr alk elution buffer (0.05 M KH_2PO_4, 1.0 M NaCl, 0.1% (v/v) Triton X-100, pH 9.2) as suggested by Hamza et al. (2009). During elution, buffer was added on top of the membranes to soak the membranes for 10 min without applying pressure. After 10 min of incubation the elution buffer was filtered through the membranes to elute viruses by applying pressure after which the concentrate was immediately neutralized using 1 M HCl. Secondary concentration was done by precipitation through addition of PEG-6000 (Sigma-Aldrich, Steinheim, Germany) and NaCl (Sigma-Aldrich, Steinheim, Germany) in a final concentration of respectively 12.5% (w/v) and 0.3 M. After overnight incubation on a shaking platform at 4°C, the concentrate was centrifuged at 10 000 x g for 30 min at 4°C. The pellet was suspended in 2 ml of PBS (Lonza) and subjected to a chloroform:butanol (C:B) purification step as previously described by Baert et al. (2008a). Briefly, 1 ml of the suspended pellet was treated with one volume of C:B (1:1, v/v) to remove inhibitory substances from the virus concentrate. The mixture was vortexed, incubated for 5 min at RT and centrifuged again at 10 000 x g for 20 min. The aqueous phase (supernatant) was isolated and stored at -80°C until RNA purification. The remaining 1 ml suspended pellet was likewise stored at -80°C for the detection of phages.

2.3.3.2. Method 2

In Method 2 (HA-SM) the same type of water pretreatment, negatively charged filter and prefilters and acid rinse were used as in Method 1. However, the elution buffer (70 ml) in this method was a 0.1% skimmed milk solution in 0.05 M glycine buffer (pH 9.5) as previously used by Wyn-Jones et al. (2011). After elution (performed as in Method 1) secondary concentration was performed by organic flocculation. The eluate was flocculated by the addition of 1 M and 0.1 M HCl until the pH reached 4.5. The resulting protein flock, containing the virus, was deposited by centrifugation at 7000 x g for 30 min at 4°C. The pellet was dissolved in 2 ml PBS after which the C:B purification step took place on 1 ml. The resulting supernatant was stored at -80°C until further RNA purification. The rest of the suspended pellet was stored at -80°C for detection of bacteriophage MS2.

2.3.3.3. Method 3

Method 3 (Zet-TGBE) was based upon the proposed method by CEN/TC275/WG6/TAG4 for viral concentration from bottled water. As such, an electropositive Zetapor membrane

(NM 14201 045SP, 3M, Meriden, CT, USA) was used in the pressure filtration system. Glass fiber filters were used as prefilters (AP20 and AP15). When the pH of the initial water was below 6.0 or exceeded 8.0, the pH was adjusted to 7.0-7.4 by adding NaOH or HCl. After filtration, 70 ml of TGBE (1%) elution buffer was added on top of the filter, followed by a 10 min incubation step. Next the elution buffer was filtered through the membranes by raising the pressure and immediately neutralized with 6 M HCl. The secondary concentration method (PEG precipitation) and subsequent C:B purification were performed as in Method 1. Samples were stored at -80°C until further processing.

2.3.3.4. Method 4

Viral concentration Method 4 (Viv-G) was based on a method previously validated by Ceeram (La Chapelle-sur-Erdre, France). The Vivaflow 50 cassettes (VF05P3, Sartorius Stedim Biotech, Goettingen, Germany) (MWCO = 50 000) were used as a tangential flow filtration system. These cassettes have a polyethersulfone membrane of 50 cm^2 active membrane area and were connected to a peristaltic pump (Masterflex L/S economy digital drive, Metrohm, Antwerp, Belgium). In case of irrigation water 2 L of spiked water was filtered (maximum speed: 100 ml/min) until a concentrated sample volume of ca. 15 ml was reached. Afterwards the cassette was rinsed with glycine buffer (0.05 M glycine, 0.15 M NaCl, pH 9.5) to have a final volume of 40 ml. After collection the pH of this 40 ml was neutralized and secondary concentration was performed by PEG precipitation by adding PEG-6000 until a final concentration of 10% (w/v) was reached. Hereupon, the overnight incubation, the concentration, the C:B purification step and subsequent storage of the samples was performed as described in Method 1.

In the case of the processing water a pre-filtration through a glass fiber filter (AP20) using the pressure filtration system was necessary before the tangential flow filtration concentration. The prefilter was eluted with 50 ml glycine buffer (contact time: 10 min) and this concentrate was pooled together with the one resulting from the subsequent tangential flow filtration.

2.3.4. Enumeration and detection of MS2 phages and MNV-1

2.3.4.1. MS2 bacteriophage enumeration

MS2 bacteriophages were quantified with the double-layer plaque assay in accordance with ISO 10705-1, using *Salmonella* Typhimurium strain WG49 as a bacterial host (Anonymous 1995).

2.3.4.2. Real-time RT-PCR for the detection of MNV-1

For nucleic acid purification the automated NucliSens® EasyMAG™ system (Biomérieux, Boxtel, the Netherlands) was utilized. After lysis of the samples (1 ml) in 2 ml lysis buffer

(Biomérieux, Marcy-l'Etoile, France) for 30 min at 56°C, the off-board protocol was used according to manufacturer's instructions. The nucleic acids were eluted in a final volume of 25 µl and stored at -80°C until further use.

Detection of MNV-1 was performed by a two-step real-time RT-PCR protocol. The reverse transcription (RT)-step, already described was performed on the undiluted and diluted (1/10 and 1/100) RNA extracts (Stals et al. 2009a) and the cDNA was stored at -20°C. Quantitative real-time PCR (qPCR) was performed according to Stals et al. (2009a) using the MNV-1 primers, probe and plasmid described by Baert et al. (2008d). Amplification data was collected and analyzed with the SDS 7300 Real-time PCR Systems' software (Applied Biosystems). A standard curve was generated using ten-fold serial dilutions (10^7 to 10 genomic copy) of the plasmid p20.3. All samples and standards were run in duplicate, except for the cDNA derived from the 1/100 diluted RNA extracts.

2.3.4.3. Detection of the indigenous contamination of water samples with GI and GII NoV

All concentrates of water samples obtained in the comparative study were reanalyzed in order to detect indigenous contamination of the tested water types with GI and GII NoV. Detection of GI and GII NoV was performed according to the in-house two-step duplex RT-qPCR described by Stals et al. (2009a). In this protocol, 3 µl of extracted RNA was used in a total final volume of 20 µl for RT-PCR. Subsequently, 5 µl was used for qPCR. The used fluorophore/quencher combinations for GI/GII NoV hydrolysis probes were respectively 6-FAM/BHQ-1 (Integrated DNA Technologies, Leuven, Belgium) and HEX/BHQ-2 (Integrated DNA Technologies).

2.3.4.4. Detection of inhibition in real-time RT-PCR

For each of the samples of the different concentration methods/water type combinations, dilutions were made of the RNA extracts up to 1/100 in nuclease free water (Qiagen, Hilden, Germany) to test for possible inhibition during the RT-qPCR. This high level of dilution and the expected Ct increases between dilutions helped ensure that PCR inhibition was not a factor in determining the recovery efficiency when the data obtained for the 1/10 diluted RNA samples was used.

2.3.5. Detection of human enteric viruses

After the comparison of four viral concentration methods for the detection of MNV-1 and MS2, the method with the best overall performance with regard to recovery efficiency over the wide range of water types tested (Method 1) was evaluated further for its efficiency in recovering relevant foodborne viruses such as NoV (GI.4 and GII.4), HAV and RV from the same five types of water used earlier (bore hole water, rain water, open well water,

river water and processing water). For the nucleic acid purification the method described earlier for the detection of MNV-1 was used with the exception that the final elution volume was 50 µl instead of 25 µl. Quantitative detection was accomplished with real-time RT-PCR.

2.3.5.1. Detection of rotavirus

For the detection of RV a two-step TaqMan® RT-qPCR assay was used, based on the one-step assay designed by Jothikumar et al. (2009) that targeted the NSP3 region of the virus genome. The reverse transcription step was performed as described by Stals et al. (2009a). All cDNA was stored at -20°C. Quantitative real-time PCR was carried out in a 25 µl reaction mix containing 5 µl of the target's cDNA and 12.5 µl of TaqMan Universal PCR Master Mix (Applied Biosystems, Branchburg, NJ, USA), which contains dUTP and uracyl N-glycosylase (UNG). The used primers and probe sequences are identical as described earlier by Jothikumar et al. (2009). The TaqMan® probe, labeled 5' FAM/3' BHQ-1 (Integrated DNA Technologies), was used at a final concentration of 100 nM, and the primers (Integrated DNA Technologies) were used at a final concentration of 250 nM (each). Real-time PCR amplifications were performed using the following conditions: incubation at 50°C for 2 min to activate UNG, initial denaturation/activation at 95°C for 10 min, followed by 50 cycles of denaturation at 94°C for 10 s, annealing at 55°C for 30 s (fluorescence data collection at the end of annealing step), and extension at 72°C for 20 s.

2.3.5.2. Detection of GI,GII NoV and HAV

In-house two-step duplex RT-qPCR protocol for detection of GI and GII NoV was performed as defined in section 2.3.4.3.

In-house two-step RT-qPCR for detection of HAV was performed using the primers and probe combination and concentrations as suggested by the CEN/TC275/WG6/TAG4 working group, for which the primers and probe were described by Costafreda et al. (2006). RT-PCR was performed according to the same protocol as for GI and GII NoV two-step detection. Quantitative real-time PCR was carried out in a 25-µl reaction mix containing 5 µl of target cDNA and 12.5 µl TaqMan Universal PCR Master Mix (Applied Biosystems, Branchburg, NJ, USA). The 5′ FAM minor groove binding TaqMan® probe (Applied Biosystems, Foster City, CA, US) was used at a final concentration of 250 nM and forward and reverse primers (Integrated DNA Technologies, Leuven, Belgium) at a final concentration of 500 and 900 nM, respectively. The qPCR conditions were as follows: 2 min at 50 °C to allow the working of UNG, 10 min at 95 °C as initial denaturation/activation step, followed by 50 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min, and extension at 65 °C for 1 min. Fluorescence data were collected at the end of the extension step.

The commercial one-step RT-qPCR detection assays for separate detection of GI, GII NoV and HAV, were named 'norovirusGI@ceeramTools®.environmental', 'norovirusGII @ceeramTools®.environmental' and 'hepatitisA@ceeramTools®.environmental' kits respectively (Ceeram, La Chapelle-sur-Erdre, France). These kits were used according to manufacturer's instructions. In these protocols 5 µl of RNA extract were used in a total volume of 25 µl reaction mixture. Primers and probes for qPCR present in these reaction mixtures are in accordance with the ones defined by CEN/TC275/WG6/TAG4. An internal amplification control (IPC) and positive and negative controls were included in each of these kits.

2.3.5.3. Absolute quantification and inhibition assessment

Absolute quantification was possible through the generation of a real-time PCR standard curve for each of the RT-qPCR detection assays described in section 2.3.5. Plasmids were used in the case of HAV, GI and GII NoV detection. For GI and GII NoV, the previously described plasmids by Stals et al. (2009a) were used. In the case of HAV, pCR 2.1-TOPO cloning vectors (Invitrogen, Carlsbad, CA, USA) containing a 174 bp PCR amplicon covering the primers-probe binding sites were kindly donated by the Belgian Scientific Institute of Public Health in Brussels (IPH, Brussels, Belgium). Tenfold serial dilutions ranging from 10^7 to 10 copies of all three plasmids were used to prepare the standard curves. In case of quantification of RV, a tenfold dilution series was used of cDNA retrieved from a 1/10 diluted feces sample of RV G1P[8] for generating the standard curve. The highest dilution giving a positive qPCR signal was assumed to be one RT-PCR unit (RT-PCRU). As such an RT-PCRU is the lowest amount of viral genomic material that can be detected when using RT-PCR. Therefore viral loadings of RV were expressed in RT-PCRU. All standard curves were generated in duplicate. Quantitative PCR was performed on the SDS 7300 Real-time PCR System (Applied Biosystems) with the inclusion of positive and negative controls. The amplification data were collected and analyzed with the SDS 7300 instruments' software.

The influence of molecular detection inhibiting substances on the performance of the different RT-qPCR assays for detection of GI & GII NoV and HAV was tested per matrix (ground water, rainwater, open-well water, river water, and processing water) by the dilution approach. In essence, this approach is focused on the Ct difference between undiluted RNA and 1/10 diluted RNA extracts; if the sample is inhibited, then the difference between Ct values of the undiluted and 1/10 diluted RNA extracts will be smaller than expected for samples not inhibited. Samples showing a ΔCt value <(slope standard curve −0.5) were considered as inhibited. RNA was maximally 1/100 diluted. The latter dilution and the expected Ct increases between dilutions ensured that PCR inhibition

was not a factor in determining the viral titer of the water samples when the data obtained for the 1/10 diluted RNA samples were used for further analysis.

2.3.6. Data analysis

The 1/10 diluted RNA samples were considered inhibitor free and were used for the calculation of the recoveries and the success rates. Except for the Method 2/open well water combination data resulting of the 1/1 dilution were used as all repeats were negative for the 1/10 diluted RNA. The recovery percentage of the spiked viruses was calculated using the following equation: percentage of recovery = the number of recovered viruses/the number of seeded viruses x 100. The success rate of a method in a specific type of water or for the detection of a specific human enteric virus was calculated using the following equation: success rate = number of repeats in which detection of the virus was possible/ number of performed repeats. For MS2, the number of viruses was expressed as plaque forming units (PFU), for NoV, MNV-1 and HAV as genomic copies (GC) and for RV as RT-qPCRU.

As the dataset was limited to three independent repeats for each method/ water type combination and human enteric pathogen/water type combination, non-parametric statistical tests were preferred. In case of the comparison of the four viral concentration methods for their efficiency in recovery of MNV-1 and MS2 for each of the five types of water, the Kruskal-Wallis (KW) test was used. In this test ranks were assigned by assigning the higher value to the better concentration method for a particular water type. The Mann-Whitney (MW) test was used to analyze the significance of the difference between the most efficient concentration methods for one water type (received the highest rank by the KW-test). The influence of the different types of water on the recovery efficiencies for MNV-1 and MS2 for each of the four methods was analyzed with the Kruskal-Wallis (KW) test too. The Friedman's test was used to compare the efficiencies of the different concentration methods for all five types of water. The Friedman's test assigned a higher rank to the more efficient method based on the performance of the method in all five types of water. Wilcoxon signed-rank (W_s) test was used to test the significance of the difference between the most efficient primary concentration methods.

In case of the evaluation of Method 1 for the detection of NoV, HAV and RV in five different types of water, the significance of the impact of the type of water on the recovery efficiency of each human enteric pathogen was analyzed according to the KW-test. The recovery efficiencies of Method 1 for MNV-1, GI and GII NoV throughout the different types of water were compared according to the Friedman's test which assigned a higher value to the more efficient recovered pathogen in all water types. The same test was

performed to compare the recovery of Method 1 for HAV, GI and GII NoV and RV throughout the different types of water.

Both the Friedman's test and the Wilcoxon signed-rank test were calculated on the median recovery efficiencies (median values not presented) for each concentration method/ water type combination or pathogen/ water type combination.

Statistical analysis was performed using SPSS software, version 19 (SPSS Inc., Chicago, IL, USA). p-Values ≤ 0.05 were deemed statistically significant.

2.4. RESULTS

2.4.1. Water sample characteristics

In the present study, two rounds of water sampling occurred from the same location for each of the five types of water involved. The first water sample was used for the comparison of different viral concentration methods for the recovery of MNV-1 and MS2 and the second water sample was used for the evaluation of the selected viral concentration method for the recovery of a broad panel of human enteric viruses including GI and GII NoV, RV and HAV. In the first round of sampling, the pH of the water samples was 6.92, 7.52, 9.06, 7.57 and 4.85 respectively for bore hole water, rain water, open well water, river water and processing water and the concentration of TSS 1.1, 0.5, 7.5, 20.1 and 133.9 mg/L respectively for each of these waters. In the second round of sampling the pH was 6.48, 8.22, 6.71, 7.24 and 7.30 respectively for bore hole water, rain water, open well water, river water and processing water and the concentration of TSS 7.6, 1.2, 28.7, 36.0 and 31.6 mg/L. For the determination of the pH three measurements were made. For the determination of the concentration of TSS three to four repeats were done for each water type.

2.4.2. Detection of MNV-1 in various types of water

The mean recovery efficiencies of the various viral concentration methods for the detection of MNV-1 in processing water and four different types of irrigation water, are summarized in Table 2.1. For all water types, the mean recovery of MNV-1 obtained with Method 1 was higher than all other methods although in only three types of water (bore hole water, open well water and processing water) this difference was statistically significant (Mann-Whitney test, $p \leq 0.05$). Method 1 also got the highest rank according to the Friedman's test ($p = 0.011$). Mean recoveries of Method 1 ranged from 4.76% for detection in open well water to 21.87% for bore hole water. The type of water had no significant impact on the recovery efficiency of MNV-1, regardless of the virus concentration method (KW-test; $p > 0.05$).

When the success rates for the detection of MNV-1 were evaluated for the four primary concentration methods, only Method 2 failed to detect MNV-1 in every repeat and was unable to detect MNV-1 at all in processing water. Methods 1, 3 and 4 enabled concentration and subsequent RT-PCR detection of MNV-1 in all 1/10 diluted RNA samples. Nevertheless, inhibition during real-time RT-PCR played an important role for these methods when the undiluted RNA samples were analyzed, especially in case of open well water, river water and processing water. The failure of detecting MNV-1 by Method 2 was probably due to the inability of the used secondary concentration method (organic flocculation) to concentrate the viruses efficiently. This was examined by spiking the elution buffer of each method with a fixed amount of genomic copies of MNV-1 and performing the correspondent secondary concentration. For the recovery of MNV-1 the secondary concentration method used in Method 2 was clearly the least effective, which can explain the lower recovery of Method 2 when compared to Method 1 (data not shown).

2.4.3. NoV detection in environmental water samples

All the water samples included in the evaluation study of the four viral concentration methods were also analyzed for the indigenous presence of GI and GII NoV. The river water sample turned out to be positive for GI and GII NoV while all other samples tested negative for both NoV genotypes. All of the tested (1/10 diluted) river water samples, obtained after concentration with the four different viral concentration methods, tested positive for GI and GII NoV, except for the samples obtained with Method 2. These samples were negative for both GI and GII NoV. As both Methods 1 and 3 started from 5 L water and subsequent dilution steps were identical, Ct values could be compared directly. The Ct-value obtained with Method 1 were 2 - 3 Ct values lower than those obtained with Method 3 when the 1/10 diluted RNA was examined (data not shown). This indicates that Method 1 had a higher recovery efficiency than Method 3. As Method 4 concentrated only 2 L river water, direct comparison of the Ct values with the results for Methods 1 and 3 (5 L sample volume) was not possible. As such, these results partially confirm the selection of Method 1 as the preferred method for further performance testing in concentration and detection of the GI and GII NoV, RV and HAV.

Table 2.1. Mean recovery efficiencies of the various concentration methods for the detection of MNV-1 in the processing water and four different types of irrigation water

Water Type	Method 1 (HA-Tr alk)		Method 2 (HA- SM)		Method 3(Zet – TGBE)		Method 4 (Viv – G)		KW-test[b] (p-value)	MW-test[c] (p-value)
	μ[a] (%) (success rate)	Range	μ (%) (success rate)	Range	μ (%) (success rate)	Range	μ (%) (success rate)	Range		
Bore hole water	21.87[(1)](3/3)	7.59-37.01	0.07 (2/2)	0.05-0.08	1.63[(2)] (3/3)	0.56-3.24	0.71 (3/3)	0.23-1.00	0.036*	0.050*
Rain water	5.78[(1)] (3/3)	2.99-10.97	nt	nt	4.96[(2)] (3/3)	0.50-8.75	0.50 (3/3)	0.16-0.89	0.113	
Open well water	4.76[(1)] (3/3)	2.37-8.19	0.001 (2/3)	0.001-0.002	0.34[(2)] (3/3)	0.24-0.44	0.36[(3)] (3/3)	0.18-0.62	0.025*	0.050*
										0.050*
River water	10.70[(1)] (3/3)	2.30-15.24	0.02 (2/3)	0.02-0.03	1.15 (3/3)	0.81-1.52	2.79[(2)] (3/3)	1.67-3.42	0.022*	0.275
Processing water	9.24[(1)] (3/3)	6.37-12.28	- (0/3)	-	0.62[(2)] (3/3)	0.50-0.75	0.44[(3)] (3/3)	0.28-0.71	0.020*	0.050*
										0.050*
									Friedman's test (p-value)	**W_s-test[d] (p-value)**
Friedman's test mean rank	4.00[(1)]		1.00		2.75[(2)]		2.75[(3)]		0.011*	0.080 0.043*

nt : not tested; '-': no detection, below detection limit;

[a] μ is the mean recovery efficiency of three values

[b] KW-test = Kruskal-Wallis test

[c] The Mann-Whitney (MW) test was done between the two most efficient concentration methods (which got the largest mean rank according to the KW-test). The mean values of these two methods are indicated with a (1) for the highest mean rank and (2) for the second highest mean rank. When the range of the method with the third highest mean rank was included or covered in the range of the method with the second highest mean rank this method's mean was indicated with (3) and two MW-test were done: (1) – (2) and (1) – (3).

[d] The Wilcoxon signed-ranks (W_s) test was done between the most efficient concentration methods (which got the largest mean rank according to the Friedman's test). The two p-values are respectively the p-values of the Wilcoxon signed rank test between the median recovery efficiencies of the concentration methods with the ranks indicated with (1)-(2) and (1)-(3).

'*' : A significant difference was detected according to the used statistical test ($p \leq 0.05$)

Table 2.2. Mean recovery efficiencies of the various concentration methods for the detection of MS2 bacteriophages in processing water and four different types of irrigation water

Water Type	Method 1 (HA-Tr alk)		Method 2 (HA- SM)		Method 3 (Zet – TGBE)		Method 4 (Viv – G)			
	μ[a] (%) (success rate)	Range	μ (%) (success rate)	Range	μ (%) (success rate)	Range	μ (%) (success rate)	Range	KW-test[b] (p-value)	MW-test[c] (p-value)
Bore hole water	5.13$^{(2)}$ (3/3)	3.49-7.14	0.02 (3/3)	0.01-0.02	17.86$^{(1)}$ (3/3)	14.52-19.76	0.03(3/3)	0.02-0.04	0.026*	0.050*
Rain water	5.57$^{(2)}$ (3/3)	3.98-6.52	nt	nt	11.03$^{(1)}$ (3/3)	9.71-12.73	0.47 (3/3)	0.08-0.26	0.027*	0.050*
Open well water	2.02$^{(2)}$ (3/3)	1.13-3.23	0.01 (3/3)	0.01-0.02	0.26 (3/3)	0.18-0.33	3.24$^{(1)}$ (3/3)	2.42-3.69	0.019*	0.127
River water	3.44$^{(2)}$ (3/3)	2.38-3.97	0.03 (3/3)	0.02-0.03	0.77 (3/3)	0.68-0.83	3.76$^{(1)}$ (3/3)	3.49-4.07	0.024*	0.825
Processing water	4.15$^{(1)}$ (3/3)	1.98-5.32	0.10 (3/3)	0.09-0.11	0.35 (3/3)	0.32-0.38	1.33$^{(2)}$ (3/3)	1.22-1.64	0.024*	0.050*
									Friedman's test (p-value)	**W_s-test[d] (p-value)**
Friedman's test mean rank	3.50$^{(1)}$		1.00		2.50		3.00$^{(2)}$		0.038*	0.138

nt : not tested;

[a] μ is the mean recovery efficiency of 3 experiments

[b] KW-test = Kruskal-Wallis test

[c] The Mann-Whitney (MW) test was done between the two most efficient concentration methods (which got the largest mean rank according to the KW-test). The mean values of these two methods are indicated with a (1) for the highest mean rank and (2) for the second highest mean rank.

[d] The Wilcoxon signed-ranks (W_s) test was done between the most efficient concentration methods (which got the largest mean rank according to the Friedman's test, indicated with respectively (1) and (2)).

'*': a significant difference was detected according to the used statistical test ($p \leq 0.05$)

2.4.4. Detection of MS2 in various types of water

The mean recovery efficiencies of the various viral concentration methods for the detection of MS2 bacteriophages in processing water and four types of irrigation water are summarized in Table 2.2. Regarding the effect of the water type on the recovery efficiency of the viral concentration method, results suggested that a subdivision could be made according to the amount of TSS in the water. Bore hole water and rain water could be grouped together as both contained a very low amount of TSS, while a second group with open well, river and processing water contained a much higher amount of TSS. This subdivision is clearly distinguishable in the recovery efficiency that was obtained with Methods 3 and 4. As expected, the type of water had a significant impact on the recovery for Method 3 (KW-test, $p=0.011$) and Method 4 (KW-test, $p=0.011$).

Regarding the primary concentration method a different method performed better for the two water type groups based on the TSS. For the group with low amount of TSS (bore hole water and rain water), Method 3 performed significantly better (MW-test, $p=0.050$). For the group with a higher amount of TSS (open well, river and processing water), Method 1 turned out to be the best option although for open well and river water Method 4 performed equally well for their recovery of MS2 (for open well water, KW-test: $p=0.019$, MW-test: $p=0.127$; for river water, KW-test: $p=0.024$, MW-test: $p=0.825$; for processing water, KW-test: $p=0.024$; MW-test: $p=0.050$). In conclusion, Method 1 gave a more consistent outcome in recovery efficiency for all water types, both for MNV-1 and for MS2, and was therefore selected as the preferred method for the overall detection of viruses in irrigation water and processing water.

2.4.5. Evaluation of Method 1 for concentration of human enteric viruses

Based on the comparison between the different viral concentration methods, Method 1 was evaluated further for the detection of HAV, GI and GII NoV and RV.

COMPARISON OF THE PERFORMANCE OF IN-HOUSE AND COMMERCIAL ASSAYS

Data from the standard curves for comparison of the in-house two-step protocol and the commercial one-step RT-qPCR assay for detection of GI, GII NoV and HAV are summarized in Table 2.3. The parameters obtained from each standard curve for each assay were not substantially different. To assess the performance of each type of assay in the presence of possible inhibitors, both assays were used in parallel to detect GI and GII NoV and HAV in all 15 concentrates derived from the evaluation of Method 1 for concentration of these enteric pathogens. No great difference in agreement was noted between the performances of both NoV RT-qPCR assays during the analysis of the 1/10 diluted RNA concentrates. On the other hand, a more clear difference in performance of the HAV RT-qPCR assays was noted, since the commercial one-step RT-qPCR detected a higher amount of genomic copies for each water sample (data not shown). A difference in measurement is probably due to a difference in performance of both RT-qPCR assays as both started from the same sample concentrate. The cause of these non-agreements in detected genomic copies is most likely a difference between both assays in their detection efficiency in the presence of inhibitors.

Assessing the effect of inhibition on the performance of the RT-qPCR assays during the analysis of each of the 15 water samples was done by comparing the Ct value resulting from the undiluted RNA sample with the respective 1/10 diluted RNA sample. Analysis of the 1/10 diluted RNA samples did not show inhibition for both assays. However slightly less inhibition was noticed in the undiluted RNA samples when analyzed with the commercial one-step RT-qPCR assay, even though the dilution of the in-house two-step RT-qPCR was 6.67 times higher (data not shown). The latter could be explained by the specific optimization of the commercial one-step RT-qPCR assay (Ceeram) for analyses of environmental samples. Hence the results using the commercial assay were further used for assessment of detection efficiency of human enteric viruses.

Table 2.3. Data from the control plasmids standard curves of the real-time RT-PCR performed for the detection of HAV, GI and GII NoV with both assays (the two-step in-house RT-qPCR and the one-step commercial RT-qPCR).

Parameters[a]	GI NoV RT-qPCR		GII NoV RT-qPCR		HAV RT-qPCR	
	Two-step in-house	One-step commercial	Two-step in-house	One-step commercial	Two-step in-house	One-step commercial
Slope	-3.32	-3.32	-3.40	-3.36	-3.48	-3.48
E_a [b]	1.00	1.00	0.97	0.98	0.94	0.94
Y intercept when X = 0.0	39.34	40.53	39.01	38.14	39.47	39.69
R^2 [c]	1.00	0.99	0.99	1.00	0.99	0.99

[a] Control plasmids were used as standard, as such viral loadings were expressed in # genomic copies detected, the tenfold dilution series were analyzed in double
[b] The amplification efficiency of the RT-qPCRs was calculated according to the formula: $E_a = (10^{-1/slope}) - 1$, where E = 1 corresponds to 100% efficiency
[c] The correlation coefficient

Table 2.4. Mean recovery efficiencies obtained for the detection of GI and GII NoV, HAV and RV in five types of water using Method 1

Volume	Water type	HAV μ[a] (%) (range) One-step RT-qPCR kit	GI NoV μ (%) (range) One-step RT-qPCR kit	GII NoV μ (%) (range) One-step RT-qPCR kit	RV μ (%) (range) Two-step RT-qPCR
5 L	Bore hole water	32.11 (21.55-37.69)	23.90 (16.06-30.02)	27.51 (22.90-32.42)	9.81 (4.79-14.26)
5 L	Rain water	29.98 (26.00-32.92)	13.17 (10.79-15.25)	19.80 (16.95-24.58)	1.31 (0.85-1.57)
2 L	Open well water	14.49 (11.53-16.32)	4.75 (3.75-5.44)	10.67 (10.12-11.40)	0.09 (0.02-0.15)
5 L	River water	21.49 (19.14-23.88)	8.33 (6.18-10.43)	13.81 (12.76-15.05)	0.03 (0.02-0.04)
1 L	Processing water	30.17 (25.10-37.99)	7.95 (3.55-12.42)	19.49 (13.99-23.82)	0.16 (0.09-0.25)
Friedman's test (p = 0.000) **mean rank**[b]		4.00	2.00	3.00	1.00
KW-test[c]		0.010*	0.002*	0.001*	0.000*

[a] μ is the mean recovery of three experiments. The success rate was 3/3 for all experiments
[b] The Friedman's statistical ranks were assigned by assigning the higher value to the viral pathogen that was detected with the highest efficiency throughout the different water types (Friedman's test, p = 0.000).
[c] Kruskal-Wallis (KW) test: when $p \leq 0.05$ the water matrix had a significant impact on the recovery of this enteric pathogen (indicated with '*')

EVALUATION METHOD 1 FOR CONCENTRATION OF GI AND GII NOV, HAV, AND RV

Results are shown in Table 2.4. The selection of Method 1 as most appropriate viral concentration method was confirmed by its high success rate (3/3 for the detection of each of the four pathogens in all five types of water). Mean recovery values for HAV ranged from 14.49% in open well water up to 32.11% in bore hole water, for GI NoV from 4.75% in open well water up to 23.90% in bore hole water, for GII NoV from 10.67% in open well water up to 27.51% in bore hole water and for RV from 0.03% in river water up to 9.81% in bore hole water. The type of water significantly impacted the recovery efficiency for each of the pathogens (KW-test, $p \leq 0.010$). The highest mean recovery for all these tested foodborne viruses – likewise to MNV-1 – was obtained in bore hole water. HAV was detected with the highest mean recovery efficiency and RV had the lowest mean recovery efficiency in each of the tested water types.

It is important to mention that, although 1-5 L of water samples were tested with each method, only a fraction of these volumes were actually analyzed by qPCR. For NoV/HAV this is caused by the fractional use of the virus extract and the purified RNA during the RNA purification and one-step RT-qPCR, respectively. In case of RV, fractional use of the cDNA in the two-step qPCR had to be taken into account as well. As such, when the initial sample volume was 5 L, the sample volume analyzed in a 5 µl real-time (RT-)PCR reaction mixture was 37.5 ml for the detection of RV and 250 ml for the detection of HAV, GI and GII NoV. Using these back volume calculations, the theoretical limit of detection (assuming recovery efficiency is 100%) was 133 GC for RV and 20 GC for detection of HAV, GI and GII NoV in 5 L water sample when the undiluted RNA sample was analyzed.

2.5. DISCUSSION

NoV and HAV in fresh produce are one of the identified virus-commodity combinations of concern according to the FAO/WHO (FAO/WHO 2008b). Lettuce can get contaminated pre-harvest by e.g. irrigation water, but also post-harvest during manipulations such as washing. In order to tackle data gabs for future viral risk assessment in the fresh produce chain, it is important to have a well evaluated concentration and detection protocol available for diverse human enteric viruses in different types of irrigation water. In literature, numerous articles have evaluated different concentration methods for the detection of human enteric viruses in multiple water sources (waste water, drinking water, river water, seawater, etc.), but this study is the first by our knowledge to expand the evaluation of different viral concentration methods including processing water.

One of the strengths of this study lays in the high amount of comparisons that were made - four viral concentration methods were evaluated for processing water and four types of irrigation water – which enabled a direct comparison of these four methods for their recovery efficiency of MNV-1 and MS2 phages in a multitude of water types. Comparison of several published reports on recovery efficiency of viral concentration methods is not straight forward as the recovery efficiency of viruses can be influenced by several other factors besides the used viral concentration method. These factors include water quality of the environmental samples (influence on the adsorption of viruses to filter media and inhibition) (Lee et al, 2011; Lewis et al, 2000; Victoria et al. 2009; Villar et al. 2006), the used PCR assay (Bofill-Mas et al. 2006), the tested sample volume and concentration of the viruses in the water sample (Li et al. 2010).

After the comparison of four viral concentration methods for the recovery of MNV-1 and MS2 phages, Method 1 was noted to be the best option to concentrate MNV-1 in the various types of water. For the detection of MS2 phages no single method performed best in all five types of water under investigation, which is in agreement with a previous study describing that different methods could be the best option for different types of water (Villar et al. 2006). When the recovery efficiencies obtained in the present study for the various viral concentration methods under investigation were compared with results obtained in the literature, these recovery efficiencies were rather low. Recovery efficiencies as high as 79% and 56% were obtained when respectively two-layer Filterite® fiberglass filters (negatively charged) and a NanoCeram virus sampler (positively charged) were used for the concentration of MS2 in tap water (Ikner et al. 2011; Scott et al. 2002). However a recent study used Method 1 based on the protocol stated in this article for the detection of MS2 (plaque assay) in 100 ml river water (spike: 10^6 PFU/100 ml) and obtained a mean recovery efficiency of 16.1% (Jones et al. 2014). The reason for the low recovery efficiency obtained in this study

can be the higher amount of water analyzed. As such in the study of Jones et al. (2014) only 100 ml of river water, with a similar high spike of MS2, was analyzed compared to the 5 L analyzed in this study. Also the type of water chemistry could be play a role. However note that in case of MS2 detection plaque assay is used for quantification, as such also the occurrence of inactivation and aggregation (Langlet et al. 2007) could play a role in these lower recovery efficiencies observed for MS2 concentration. Nevertheless in this study Method 1 showed a consistent outcome for the detection of MS2 in all types of water and was the best option for the detection of MNV-1. Therefore this method was selected as the most appropriate method for further evaluation to concentrate foodborne viruses in processing water and the various types of irrigation water.

It was demonstrated that the viral concentration method called 'Method 1' is an effective way of concentrating a broad panel of human enteric viruses, including GI.4 and GII.4 NoV, RV and HAV, from medium volumes of these various water types. Even though water quality within the relevant water types can be hugely variable, the consistent performance of Method 1 for all tested types of water is a good indication of the broad applicability of this method for further use. Although the recovery efficiency for RV was in most cases lower compared to the NoV and HAV, no single combination of adsorbent/adsorption conditions was expected to give optimum efficiencies for all virus types from all water types (Sobsey and Glass 1984; Wallis et al. 1979). Previously Fumian et al. (2010) experienced also a low recovery efficiency for the detection of RV when using the method described by Katayama et al. (2002). A mean recovery of 3.5% (range: 1.5-5.5%) was obtained for the detection of RV in 42 ml of raw sewage (diluted in 2 L of distilled water to avoid clogging).

Interestingly, the recovery efficiencies of Method 1 for MNV-1, GI and GII NoV throughout the different types of water were not significantly different (Friedman's Test, $p = 0.124$), demonstrating that MNV-1 is an appropriate NoV surrogate and can be used as a process control for NoV detection in these water types. This is in accordance with other studies that have suggested MNV-1 as a good surrogate for human NoV detection in water (Gibson and Schwab 2011; Lee et al. 2011). Despite the positive evaluation of MNV-1 as a surrogate for human NoV in several studies (Bae and Schwab 2008; Cannon et al. 2006), other surrogate viruses such as MS2 phages (Blaise-Boisseau et al. 2010), feline calicivirus (FCV) (Mattison et al. 2009; Steyer et al. 2011), mengovirus (da Silva et al. 2007) and bacteriophage PP7 (Fumian et al. 2010) have been used as process control for the concentration and detection of viruses in water.

When comparing the recovery efficiencies obtained in this study for Method 1 to other studies, it is important to keep in mind that the obtained recoveries are the combined recovery rate of the VIRADEL and the PEG-6000 precipitation step from 1 to 5 L water samples as the

efficiencies of secondary concentration methods can differ significant (Lee et al. 2011; Wu et al. 2011). The mean recovery efficiency for detection of GII NoV in this study was 13.81% in river water and 27.51% in bore hole water, which is similar to other studies using electronegative membranes that found recovery efficiencies of 15% (Haramoto et al. 2009) and 17.8% (Victoria et al. 2009) in river water samples of respectively 250 ml and 2 L. Other methods based on glass wool obtained recovery efficiencies for GII NoV of approx. 3.4% in fresh water (10 L) (Albinana-Gimenez et al. 2009) and 21-45% (20 L) in ground water (Lambertini et al. 2008).

When back volume calculations were done for the detection of human enteric viruses, the sample volume analyzed in a 5 µl (RT-) qPCR reaction mixture seems low. But the analysis of a larger sample volume (> 5L) does not necessarily result in a higher sensitivity as suspended materials and inhibitors are also concentrated with viral particles (Albinana-Gimenez et al. 2009; Hata et al. 2011). This was demonstrated in a study by Albinana-Gimenez et al. (2009) in which a lower concentration of the virus was found when 50 L of Llobregat river water was tested compared to when 10 L were tested. This stresses the importance of further research to reduce this molecular inhibition in order to make full use of the potentially higher sensitivity of water concentration methods that can process large volumes of water. Remark also that in the current study relatively high spikes were used in the water. Ideally further assessment of Method 1 should include a dilution series of spikes to be tested to determine the practical limit of detection of Method 1.

Concerning the evaluation of the in-house two-step RT-qPCR assays versus the commercial one-step RT-qPCR assays for detection of GI, GII NoV, and HAV, the use of the two-step RT-qPCR assays in research remains justified as the synthesized cDNA can be used for confirmation and/or genotyping purposes (Lees 2010), and only minor differences were observed between both RT-qPCR assays. However, the use of a commercially available RT-qPCR has other advantages such as the possibility for highly standardized work, and this one-step protocol reduces the cross-contamination potential and the work load. As such, the two-step RT-qPCR assay requires - in comparison to the one-step approach - an additional hands-on time of approximately 6 h. In addition, the use of non-commercial protocols requires more knowledge and experience than the use of an all-in-one commercial kit, which makes these kits easier to be used in routine labs.

In conclusion, Method 1 which is based on the VIRADEL principle using an electronegative HA-filter in combination with an elution buffer earlier optimized by Hamza et al. (2009) was evaluated as the best option for the detection of enteric viruses in a broad range of sources of irrigation water as well as processing water from fresh-cut produce companies. Although the recovery efficiency was influenced by the type of virus and the type of water, overall the

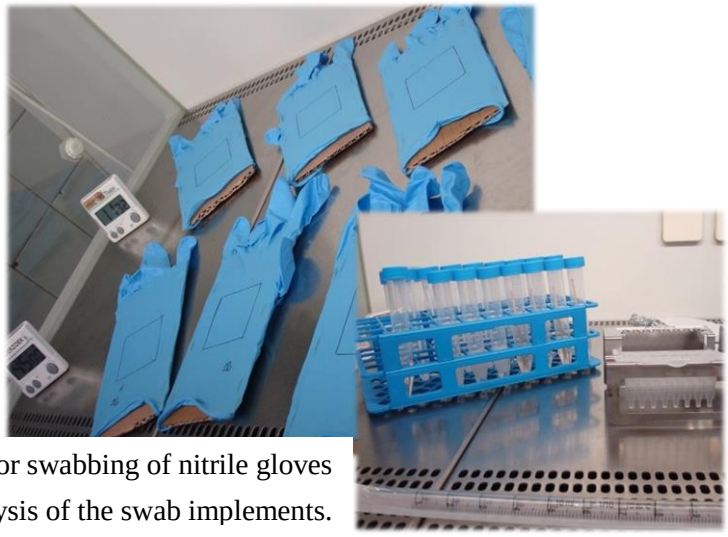
success rate of Method 1 for detection of human enteric viruses HAV, GI and GII NoV and RV was 3/3 in all water types. However, for RV the recovery efficiency was <2% in all included water types, except for bore hole water (mean recovery efficiency of 9.8%). As such Method 1 appeared to be less suited for the concentration of RV. Real-time RT-PCR was used for the detection of MNV-1, GI.4 and GII.4 NoV, RV and HAV. Currently real-time PCR is still the method of choice even though it is not possible to discriminate between viable and non-viable viruses (Croci et al., 2008). But these techniques are useful for monitoring environmental contamination, unraveling possible transmission routes and possible source tracking when an outbreak occurs.

CHAPTER 3

SEMI-DIRECT LYSIS OF SWABS AND EVALUATION OF THEIR EFFICIENCIES TO RECOVER HUMAN NOROVIRUSES GI AND GII FROM SURFACES

Redrafted from

De Keuckelaere, A., Stals, A., Uyttendaele, M. (2014). Semi-direct lysis of swabs and evaluation of their efficiencies to recover human noroviruses GI and GII from surfaces. Food and Environmental Virology 6 (2), 132-139.



Experimental set-up for swabbing of nitrile gloves and lysis of the swab implements.

Authors contributions

Conception and interpretation of the paper was done by Ann De Keuckelaere, Ambroos Stals, and Mieke Uyttendaele. The planning, practical lab work, and writing was done by Ann De Keuckelaere.

3. SEMI-DIRECT LYSIS OF SWABS AND EVALUATION OF THEIR EFFICIENCIES TO RECOVER HUMAN NOROVIRUSES GI AND GII FROM SURFACES

3.1. ABSTRACT

Enteric viruses such as noroviruses (NoV) continue to be the cause of widespread viral outbreaks due to person-to-person transmission, contaminated food and contaminated surfaces. In order to optimize swabbing methodology for the detection of viruses on (food) contact surfaces, three swab elution/extraction strategies were compared in part one of this study of which one strategy was based on the recently launched ISO protocol (ISO/TS 15216-1) for the determination of hepatitis A virus and NoV in food using real-time RT-PCR (RT-qPCR). These three swab elution/extraction strategies were tested for the detection of GI.4 and GII.4 NoV on high-density polyethylene (HD-PE) surfaces with the use of cotton swabs. For detection of GI.4 and GII.4, the sample recovery efficiency (SRE) obtained with the direct lysis strategy (based on ISO/TS 15216-1) was significantly lower than the SRE obtained with both other strategies. The semi-direct lysis strategy was chosen to assess the SRE of two common swabs (cotton swab and polyester swab) compared to the biowipe (Biomérieux, Lyon, France) on three surfaces (HD-PE, neoprene rubber and nitrile gloves). For both surfaces HD-PE and nitrile gloves, no significant differences in SRE of GI.4 and GII.4 NoV were detected between the three different swabs. For the coarser neoprene rubber, biowipes turned out to be the best option for detecting both GI.4 and GII.4 NoV.

3.2. INTRODUCTION

Monitoring surface hygiene is a well-known quality control measurement within the food industry. Surfaces are not only swabbed for the traditional hygiene assessment (based on total aerobic count), but also for the detection/quantification of bacterial pathogens, allergens, and ATP bioluminescence as an alternative measure for surface hygiene, and viruses (Moore and Griffith 2002; Wang et al. 2010; Daelman et al. 2013; Boxman et al. 2011). Environmental surfaces are in fact a well-known transmission route for (foodborne) viral outbreaks (Boone and Gerba 2007; Cheesbrough et al. 2000; Isakbaeva et al. 2005; Patterson et al. 1997; Stals et al. 2013a).

Recent studies have been positive about the use of environmental swabs for the detection of enteric viruses in food producing areas and healthcare centers (Boxman et al. 2011; Carducci et al. 2011; Wu et al. 2005). While detection methods for viruses have only recently become available in the area of food safety, in medical healthcare the usage of swabs for the detection of viruses is a well-established tool to take clinical samples (Green et al. 1998, Nakanishi et al. 2009) and surface samples in hospital settings (Carducci et al. 2002, 2011; Wu et al. 2005). The use of swabs on food contact surfaces for the detection of (pathogenic) bacteria is well known and has resulted in the International Standard ISO 18593, describing surface sampling methods for the detection or enumeration of bacteria in food processing area and equipment (Anonymous 2004) and continues to be a topic of further research as different swabs and (food) surfaces remain to be tested (Moore and Griffith 2007; Hedin et al. 2010; Lutz et al. 2013). However, only a limited number of studies regarding the recovery of enteric viruses (or surrogates) on food (contact) surfaces has been published (Scherer et al. 2009; Taku et al. 2002; Julian et al. 2011; Jones et al. 2012; Rönnqvist et al. 2013) and a new ISO protocol (ISO/TS 15216-1) (Anonymous 2012c) has only recently been adopted as a technical specification with a sampling procedure for the detection of hepatitis A virus and norovirus on food surfaces.

As such, the presented study (1) provides new data on the comparison of different swab elution/extraction strategies, of which one closely resembles the protocol suggested in the ISO/TS 15216-1, and (2) provides data on the efficiency of three different swab implements for environmental sampling of enteric viruses on different surfaces frequently found at the farm level during harvesting (gloves, transport rubber bands, plastic crates) using the semi-direct lysis method (strategy 3) which is similar as the one recently described by Rönnqvist et al. (2013). In contrast to other studies, no NoV surrogate viruses were used for determining the sample recovery efficiency (SRE) (Julian et al. 2011; Taku et al. 2002; Herzog et al. 2012) on different surfaces. As actual quantitative data on norovirus detection on surfaces by swabbing is scarce and comparing data from one swab

study to another is challenging due to the large number of parameters influencing the SRE, this study aimed to compare three swab implements (cotton swab, polyester swab and biowipes) in their efficiency for the detection of GI.4 and GII.4 noroviruses (NoV) on three different test surfaces (neoprene rubber (NR), high-density polyethylene (HD-PE) and nitrile gloves (GL)).

3.3. MATERIAL AND METHODS

3.3.1. Surfaces and swabs

The three surfaces used in this study were HD-PE, NR and powder free blue (PF 240) nitrile gloves (GL) (Shield Scientific, B.V., Malaysia). These surfaces are likely to come in contact with fresh produce during harvesting or further processing. For the HD-PE and neoprene surfaces, areas of 100 cm² were denoted and prior to each experiment these surfaces were decontaminated by the use of antiviral RBS Viro spray (Sigma Aldrich, Steinheim, Germany) and soaking (10 min) and rinsing with boiling water. In case of the nitrile gloves, areas of 25 cm² were marked after the insertion of a piece of carton in the glove and administration of clamps to keep the surface in a stretched position. No decontamination prior to the inoculation experiment was performed as each glove was only used once. The three sterile swab implements under study were the cotton swab (150C) (Copan, Italy), the polyester swab (159C) (Copan, Italy) and the recent biowipe (Biomérieux, Lyon, France). Biowipes (2.5 by 3.5 cm) (Biomérieux) are composed of a mixture of fibers and microfibers (cotton, polyester and polyamide fibers) wetted in PBS buffer (pH 8.0).

3.3.2. Virus stocks

Both NoV GI.4 and GII.4 stool samples were kindly provided by the National Institute for Public Health and the Environment (RIVM, Bilthoven, the Netherlands). 10% suspensions in phosphate-buffered saline (PBS, pH 7.2, Lonza, Verviers, Belgium) of both stocks were diluted (final dilution was approx. 1% of the initial stool sample for each pathogen) and eventually mixed until a stock concentration of approx. 3×10^4 genomic copies of GI.4 NoV/100 µl and approx. 9×10^4 genomic copies of GII.4 NoV/100 µl was obtained for experiment part 1 in which different swab elution/extraction strategies were compared in their SRE of NoV on HD-PE. In part 2, in which the SRE of different swab implements was obtained for the detection of NoV on different surfaces, the stock concentration contained approx. 2×10^5 genomic copies GI.4 NoV/100 µl and approx. 1×10^5 genomic copies GII.4 NoV/100 µl. Viral stocks were quantified by molecular methods using a standard curve as described in paragraph 3.3.4. Aliquots of this stock concentration were stored at -80°C until use.

3.3.3. Study design

Demarcated areas on each surface were spiked with 100 µl NoV suspension in case of HD-PE and NR and 25 µl in case of the nitrile gloves (GL). The viral stock solution was administered in little droplets on the surface and smeared all over the surface with a sterile inoculation pin, after which the surface was allowed to dry for 45 min in a biosafety cabinet.

During one experiment 12 surfaces of the same material (either HD-PE, NR or GL) were spiked on three subsequent inoculation times (3 batches) (Figure 3.1.). For each batch one random surface was spiked with NoV-free PBS, which acted as negative control surface, and three surfaces were spiked with the described viral stock solution. In one batch, each of the three types of swab elution/extraction strategies or types of swabs was used to randomly analyze one of the three spiked surface. Each surface was swabbed in three directions - horizontal, vertical, and diagonal - in such a way that the whole surface came each time in contact with the whole swab. During one experiment each swab elution/extraction strategy or type of swab implement was used to analyze one of the three surfaces used as negative control surface and 100 µl of the spike was extracted for calculation of the SRE.

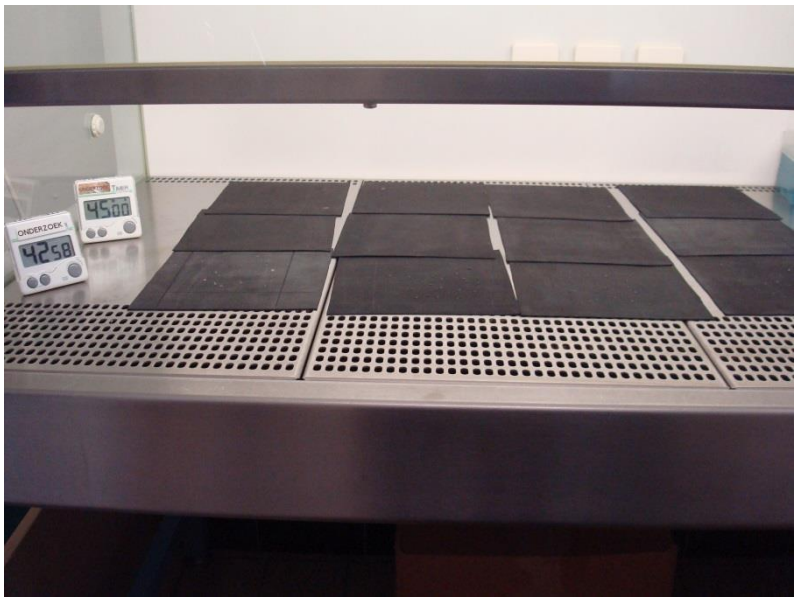


Figure 3.1. Picture of experimental study design for comparing the SRE of the different swab implements on neoprene rubber. Each of the three batches of surfaces (the different rows) were spiked on three subsequent inoculation times in order to avoid to many surfaces that needs to be sampled after 45 min of drying time.

3.3.3.1. Set-up Part 1: Comparison of different swab elution/extraction strategies

For the comparison of different swab elution/extraction strategies, the cotton swabs were used on 100 cm² HD-PE surfaces. During each of the three batches one surface was

swabbed according to each of the following strategies. *Strategy 1*: the swab was moisturized only once in the beginning in PBS and the demarked area was swabbed according to the above stated protocol. Afterwards the used swab was stored in an empty 15 ml centrifuge tube on ice until the lysis step and the RNA extraction process. *Strategy 2*: the swab was moisturized each time before swabbing the surface in a different direction (horizontally, vertically, diagonally) by dipping in 1.0 ml PBS in a 15 ml centrifuge tube and afterwards removing excess liquid by pressing the swab against the wall. This dipping and pressing cycle was repeated each occasion three times. At the end of the swabbing the swab was eluted in the PBS by 60 sec vortexing and pressing the swab against the wall to remove excess liquid. The swab was removed and the swab eluate (in the 15 ml tube) was stored on ice until the lysis step. *Strategy 3*: likewise as in strategy 2, the swab was moisturized before swabbing the surface in each of the three directions. This time 0.5 ml of PBS was used in a 15 ml centrifuge tube. After swabbing the swab was stored in the liquid, on ice until the lysis step. The maximum storage time of the samples on ice was approx. 2.5 h, before the start of the lysis step.

The lysis step for all three strategies involved of the addition of different amounts of the NucliSENS easyMAG lysis buffer (BioMérieux, Boxtel, The Netherlands), namely, 3, 2, and 2.5 ml in cases of strategies 1, 2 and 3, respectively, and subsequent incubation of 10 min at room temperature (RT) after a short mixing by vortexing. The lysis step took place immediately after the swabbing of the 12 surfaces during one experiment. For strategy 1, this lysis step resulted in a direct lysis method of the swab itself which is in accordance with the protocol suggested in the recently released ISO method for the detection of NoV in food using real-time RT-PCR (ISO/TS 15216-1:2012(E)). The exact protocol as stated in the current ISO/TS 15216-1 method was not included as this experiment predates the arrival of the ISO/TS 15216-1 method. However, this protocol diverged only to a small extent compared to the new standard protocol: in our case, the swab was lysed for 10 min, whereas a simple immersion and pressing cycle should be repeated 3-4 times according to the ISO/TS 15216-1. For strategy 2 the lysis buffer was added to the eluate and for strategy 3, this protocol resulted in a semi-direct lysis method as both the eluate and swab (present in one tube) were lysed by the addition of 2.5 ml of lysis buffer. After incubation (10 min, RT), the lysis buffer was removed and RNA extraction was performed using the automated NucliSens® EasyMAG™ system 2.0 (Biomérieux, Boxtel, the Netherlands), following generic 2.0.1 protocol for off-board lysis incubation according to manufacturer's guidelines. During each run of the automated NucliSens EasyMAG one well was reserved as negative control (addition of 500 µl PBS) to control for cross-contamination and contaminated reagents. The final elution volume was 25 µl which was adequately stored at -80°C.

3.3.3.2. Set-up Part 2: Comparison of SRE of different swab implements tested on different surfaces

The SRE obtained with cotton swabs, polyester swabs and biowipes for the detection of GI and GII NoV on three different surfaces (HD-PE, GL, and NR), was explored in Part 2. Swab elution/extraction strategy 3, the semi-direct lysis method, was used for the cotton swabs and the polyester swabs because of the positive results in Part 1 and because this strategy closely resembles the elution/extraction strategy for the biowipes (which was done according to manufacturer's guidelines). As such, in case of the biowipe, moistening in PBS was not necessary in the beginning and in between swabbing of different directions as these biowipes were stored in moisturized condition in their individual wraps. Before the usage of a new biowipe, fresh gloves were administered as these biowipes came in direct contact with the gloves during swabbing. After swabbing the used biowipe was stored in a 15 ml centrifuge tube on ice until the lysis step. In case of the biowipe 3 ml of lysis buffer (Biomérieux) was added. After 10-min incubation at room temperature, the lysis buffer was added to the sample strip of the NucliSENS EasyMAG after which the off-board protocol was followed as previously stated.

For each of the three surfaces the described experiment was repeated two times, separate in time, each with triplicate samples per swab implement type. In total six repeats were performed for every surface/swab combination, which were combined in one data unit. To reduce operator bias (due to e.g. a difference in applied pressure), the same person carried out all the experiments.

3.3.4. Real-time RT-PCR

The two-step RT-qPCR was performed as described in Stals et al. (2009a). The RT-step was performed twice for each sample: once for the undiluted RNA extract and once for the 1:4 diluted RNA extract. Each time 3 µl RNA was included in a total volume of 20 µl of reaction mix. All cDNA preparations were stored at -20°C.

The qPCR assay was used as a duplex qPCR for the detection of GI and GII NoVs. For real-time quantification, 5 µl of template cDNA was included in 25 µl of reaction mix, and was performed on the SDS 7300 Real-time PCR System (Applied Biosystems). Tenfold serial dilutions ranging from 10^7 to 10^1 copies of the control plasmids for GI and GII NoVs described by Stals et al. (2009a) were used to prepare the standard curves. Standard curves were performed in duplicate, and amplification data were collected and analyzed using the SDS 7300 instrument's software. Aliquots of the spike were analyzed in quadruplicate (Part 1) or duplicate (Part 2). Mean values were used for calculation of the SRE. The used fluorophore/quencher combinations for GI and GII NoV probes were 6-FAM/BHQ-1

(Integrated DNA Technologies, Leuven, Belgium) and HEX/BHQ-2 (Integrated DNA Technologies), respectively.

3.3.5. Data analysis

Inhibition assessment was performed by the dilution approach. In essence, this approach is focused on the Ct difference between undiluted RNA and in this case 1:4 diluted RNA extracts. Samples showing a ΔCt between 2.0 – 0.2 and 2.0 + 0.2 were considered as inhibitor-free, and in this case the recovery efficiency was calculated using the data obtained with the undiluted RNA extract. If ΔCt < 1.8, then the recovery efficiency was calculated using the data obtained with the 1:4 diluted RNA extract. The sample recovery efficiency (SRE) of the spiked viruses was calculated using the following equation: percentage of recovery = the number of recovered viruses/the number of seeded viruses x 100.

In order to perform the correct statistical test, the normality was checked each time using the Shapiro-Wilk test, and the equality of variances was tested with the Levene's test. When both assumptions were met, ANOVA was used to define significant differences. In case of a significant difference, the Bonferroni test was used as post hoc multiple comparison test. When the assumptions for ANOVA were not met, the Kruskal-Wallis (KW) test was used, and when significant differences were found, the applied post hoc tests were Mann-Withney (MW) tests with the use of a Bonferroni correction. Statistical analysis was performed using SPSS software, version 20 (SPSS Inc. Chicago, IL, USA). p-Values ≤0.05 were deemed statistically significant.

3.4. RESULTS

3.4.1. Comparison of different swab elution/extraction strategies

The mean SRE and standard deviation for the detection of GI and GII NoV on HD-PE according to the three different swab elution/extraction strategies using a cotton swab are depicted in Figure 3.2. For the detection of both GI and GII NoV, there was a significant difference in recovery efficiencies obtained with the three swab elution/extraction strategies (ANOVA, p=0.002 for GI, p=0.001 for GII). For both viruses, strategy 1 was significantly less efficient (Bonferroni, p <0.020) than strategies 2 and 3, resulting in mean recovery efficiency values of 27.0% ± 26.5% and 18.9% ± 14.3% for GI and GII NoV, respectively. For both strains, there was no significant difference in SRE obtained with strategy 2 or 3 (Bonferroni, p=0.836 for GI, p=0.073 for GII). Both strategies obtained high recovery efficiencies (SRE >70%). Mean recovery efficiencies >100% can be attributed to measurement uncertainty on the determination of the number of genomic copies, and has been observed in the literature (Julian et al. 2011; Stals et al. 2011b). Due

to measurement uncertainty the genomic copies detected in the spike used in Part 1 (spike was analyzed in quadruplicate), for example, consisted of a mean value and a standard deviation, out of which the mean value was used for calculation of the SRE.

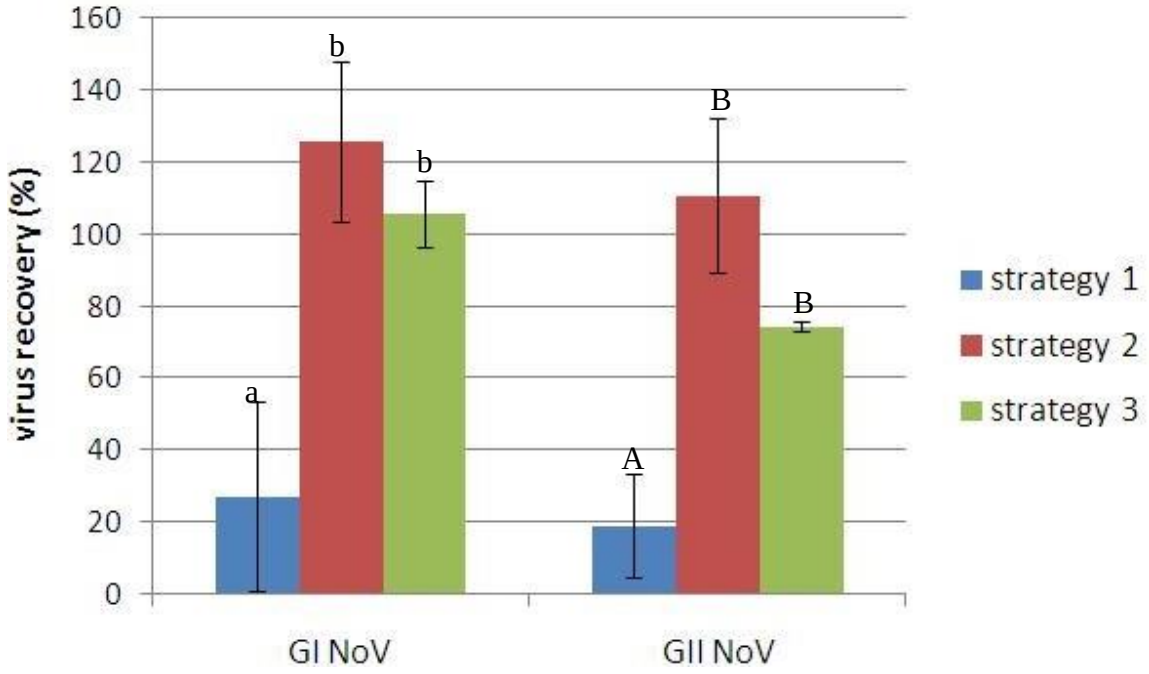


Figure 3.2. The mean sample recovery efficiencies for the detection of GI and GII NoV on HD-PE according to three different swab elution/extraction strategies. Error bars represent the standard deviation. For each spike, means (n=3) marked with the same letter are not significantly different.

3.4.2. Comparison of recovery efficiencies of different swab implements tested on different surfaces

DETECTION OF GI NOV

For the detection of GI NoV (Figure 3.3.), the SRE (independent of the type of swab implement) was significantly dependent on the type of surface tested (KW-test, p=0.000). As such, the SREs for GI NoV detection on both HD-PE and gloves were significantly higher than that for GI NoV detection on NR (MW-test, p <0.008). No significant difference in SREs was detected between HD-PE and gloves (MW-test, p=0.066).

When the performance of the three different swab implements under study (cotton swab, polyester swab and biowipe) were compared for each test surface, no significant difference in SREs was observed on the surfaces of HD-PE (ANOVA, p=0.125) and GL (ANOVA, p=0.094). On the coarser surface of NR, the use of biowipes (SRE = 41.3% ± 12.4%) resulted in a significant higher recovery efficiency than when cotton swabs (SRE = 13.2% ± 5.2%) were used for the detection of GI NoV (KW-test, p=0.007; MW-test, p<0.008). However, no significant difference in the performance of biowipes and polyester swabs

(SRE = 19.8% ± 12.4%) could be detected (MW-test, p=0.010), although the mean SRE obtained with biowipes was considerably higher than the SRE obtained when using polyester swabs.

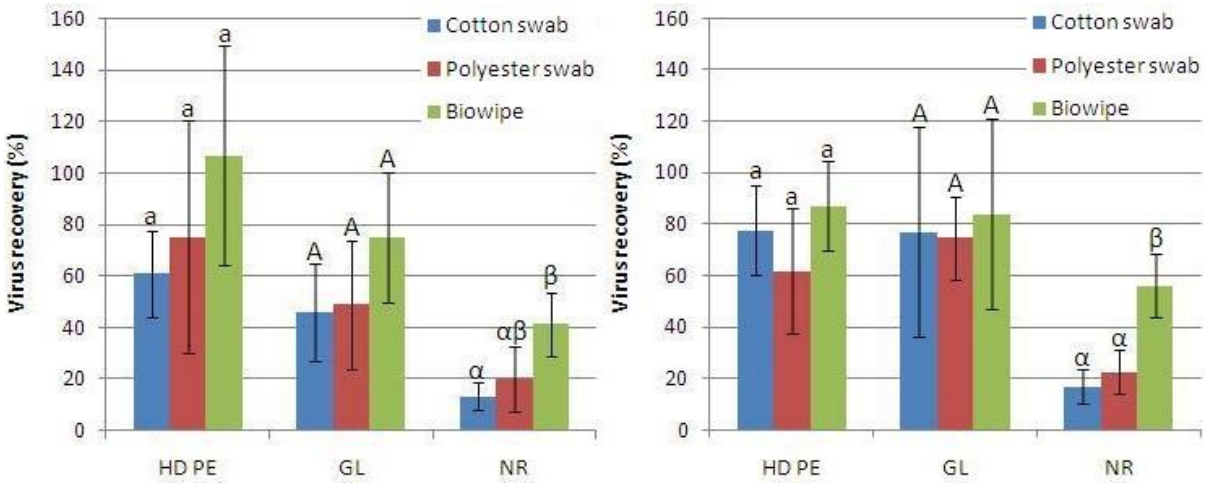


Figure. 3.3. The mean sample recovery efficiency (SRE) (%) for detection of GI NoV (left) and GII Nov (right) when three different swab implements were used on three different surfaces: HD-PE: high-density polyethylene; GL: nitrile gloves; NR: neoprene rubber. Error bars represent the standard deviation. For each type of surface, means (n=6) marked with the same letter are not significantly different.

DETECTION OF GII NOV

For the detection of GII NoV (Figure 3.3), the SRE was likewise significant depending on the type of surface tested (KW-test, p=0.000). As in the case of the detection of GI NoV, for GII NoV, similarly, no significant difference in SREs was detected between the surfaces HD-PE and gloves (MW-test, p=0.000). However, both previous surfaces obtained a significant higher SRE for GII NoV than obtained for NR (MW-test, p=0.963). For GII NoVs, no significant difference was observed in the performance of the three tested swab implements on HD-PE and on gloves (ANOVA, respectively p=0.144 and p=0.881). In the case of NR, there was a significant difference between the SREs obtained with the different swabs (ANOVA, p=0.000). In this case, the biowipe (SRE = 56.1% ± 12.5%) performed significantly better than both the cotton swab (SRE = 16.9% ± 6.6%) (Bonferroni, p=0.000) and polyester swab (SRE = 22.5% ± 8.7%) (Bonferroni, p=0.000). Cotton swab and polyester swab performed equally (Bonferroni, p=0.979). Significant differences in SREs between both viral strains (GI and GII NoV) on the different surfaces were observed only for the GL (t-test, p=0.027). For both other surfaces, HD-PE (MW-test, p=0.696) and NR (MW-test, p=0.335), no significant difference in SREs between both viral strains could be detected.

INHIBITION CONTROL

In this study the dilution approach was used to assess inhibition of the RT-qPCR detection step, instead of the use of an RT-PCR control as suggested by ISO/TS 15216-1. The choice to use the dilution approach was based on the results obtained when MNV-1 RNA was added as an RT-PCR control to a subset of the samples of this experiment Part 2. An MNV-1 RT-PCR control was added to the undiluted and 1:4 diluted RNA extracts of half of the repeats of each swab/surface combination as described earlier (Stals et al. 2011c). When the inhibition assessment according to the ISO/TS 15216-1 proposal (RT-PCR control recovery efficiency >25% = no inhibition) was performed and compared to the inhibition assessment obtained with the dilution approach, the latter was judged more sensitive, as according to the RT-PCR control approach, all samples (1:1 and 1:4 diluted RNA) were not inhibited, while according to the dilution approach in approximately half of the undiluted samples, some level of inhibition ($\Delta Ct < 1.8$) played a role. As such, due to the detected inhibition when using the dilution approach in approx. half of the samples, the SREs obtained with the 1:4 diluted RNA were used, which led to higher SRE compared to those obtained when inhibition was assessed according to the ISO/TS 15216-1 protocol which used >25% as threshold for recovery efficiency of the RT-PCR control (data not shown). Diluting of RNA until obtaining a 1:4 ratio was judged sufficient, as inhibition of the undiluted RNA extract was minor according to the dilution approach and non-existing in the undiluted and 1:4 diluted RNA according to the threshold level of >25% recovery efficiency of the RT-PCR control proposed by the ISO/TS 15216-1 protocol.

3.5. DISCUSSION

Next to traditional bacteriology analysis of surfaces, swabbing for the detection of enteric viruses is increasingly being used during outbreak investigations (Wadl et al. 2010; Cheesbrough et al. 2000; Repp et al. 2013; Boxman et al. 2009) and in environmental research studies (Boxman et al. 2011; Akhter et al. 1995; Russell et al. 2006). Contaminated (food) surfaces have led/contributed in the past to widespread/prolonged NoV outbreaks (Isakbaeva et al. 2005; Repp et al. 2013; Patterson et al. 1997; Evans et al. 2002; Kuusi et al. 2002). As such, swabbing for enteric viruses such as NoV, has proven not only to be useful during outbreak investigations, but it could also play a role in prevention strategies by means of the analysis of critical control points during food preparation. However, people should be careful regarding the interpretation of swab sample data. One should consider a positive swab sample as an indicator of surface contamination, which implies a potential risk of exposure, whereas negative swab samples do not completely assure absence of infectious particles and hence the absence of the potential risk of exposure (Scherer et al. 2009). Compliance with Good Hygienic Practices

(GHP) and the adherence to precautionary principles in case of an infected food handler are still advised.

A first ISO/TS protocol for the determination of hepatitis A virus and NoV in food using RT-qPCR has been launched recently (ISO/TS 15216-1:2012), including a section for the swabbing of food surfaces and the use of appropriate controls to prevent false positive results due to cross-contamination and false negative results due to inhibition of the molecular detection assay. It should be noted that the latter ISO protocol is until today still a technical specification, meaning that improvements to the proposed protocols can still easily be inserted, and as such further research into these detection protocols can contribute to a better proposal, and over a period of time, an optimized international standard. As research on different swabbing techniques/swab extraction techniques for the detection of enteric viruses is rare, this study aimed to contribute to this research topic.

In the first part of this study three different swab elution/extraction strategies were compared. Both strategies 2 and 3 provided high SREs and performed significantly better than strategy 1 for the detection of GI and GII NoV on HD-PE, despite the limited differences between the design of strategy 1 and 3. Their differences in recovery efficiencies may be due to the differences in the storage conditions after swabbing which was in liquid state in case of strategy 3, although the applied storage time was rather limited in this study (max. 2.5 h on ice). A second hypothesis is that the repeated wetting of the swab itself between swabbing directions in strategy 3 improved the recovery/removal of the dried NoV from the surface: as when strategy 1 was applied, the swab seemed desiccated at the end of the swabbing of the larger 100 cm² surfaces. The application of more moisture before swabbing has been proven to be beneficial for the recovery of bacteriophage P22 on different surfaces (Herzog et al. 2012). In Herzog et al.'s (2012) study, the addition of a wetting step before swabbing with a pre-moistened antistatic wipe, had resulted in the SRE values being doubled in a majority of the cases. In case of viral transfer efficiency, a wet acceptor surface (e.g. lettuce) has been proven to be more effective for viral transfer than a dry acceptor surface in case of the transfer of Feline calicivirus from stainless steel (donor surface) towards lettuce (acceptor surface) (D'Souza et al. 2006). Both previous studies show (indirectly) that the difference in the degree of moisture on the swab could lead to a different SREs for the detection of NoV on HD-PE.

As such, due to the significant lower values of SRE obtained with strategy 1, which is in close agreement with the new ISO/TS 15216-1 protocol, one can question the efficiency of the method proposed in this new ISO/TS for the detection of NoV on larger (100 cm²) surfaces.

Strategy 3 was chosen over strategy 2 as it was our goal (in Part 2) to compare common swabs such as cotton swab and polyester swab against the biowipe (Biomérieux) for which the protocol also included direct lysis of the biowipe in lysis buffer. The elution/lysis technique applied in strategy 3 is almost equal to the one recently applied by Rönnqvist et al. (2013), although both studies were performed independently. As such, this semi-direct lysis strategy applied for the cotton and polyester swab in Part 2 differs significantly from other studies, as in this study the swab itself, together with the eluate, is subjected to the lysis step.

When viewing the mean recovery efficiencies for the different swab implements on different surfaces (Figure 3.2.), a relatively high standard deviation (SD) can be noted, even though the surface/swab combination stays constant, and all experiments were carried out by the same person. This variability in recovery efficiencies for swabbing was also noted in previous studies (Scherer et al. 2009; Rönnqvist et al. 2013; Moore and Griffith 2002) and can be due to a variety of factors, both inherent and extrinsic to the used sampling mechanism. Influencing factors inherent to the sampling mechanisms are e.g. the swab material and the mechanical removal action. Factors extrinsic to the used sampling mechanism are e.g. inhomogeneous surface deposition and variable attachment of virus to the surface (Scherer et al. 2009). In spite of this huge variability in recovery efficiencies, biowipes had the intensity to perform better on coarser surfaces such as the NR, than cotton swabs and polyester swabs. On other surfaces (HD-PE and gloves) no significant differences could be detected in the performance of the different swabs although the recovery efficiencies obtained with the biowipes were consistently higher than those obtained with cotton swabs and polyester swabs. The predomination of microfiber cloth over polyester and cotton swabs for the detection of viral RNA was previously observed by Julian et al. (2011).

In comparable studies, such as detection of GII.3 NoV on HD-PE with the use of a cotton swab performed by Scherer et al. (2009) or the detection of MS2 RNA on PVC plastic using a cotton swab performed by Julian *et al.* (2011), the obtained mean recovery were respectively, 33.1 ± 23.3% and 7 – 13% (depending on the eluent type) which is considerable lower to the 77.5 ± 17.5% recovery obtained in this study. Despite this difference, direct comparison is difficult because of other differences besides the swab elution/extraction strategy such as incubation time and type of virus. When the results obtained in this study for detection of GII.4 on HD-PE with cotton (77.5% ± 17.5%) and polyester-tipped swabs (61.6% ± 24.4%) are compared with those obtained by Rönnqvist et al. (2013) (in both cases SRE = ca. 30%) on low-density polyethylene, again a higher recovery was noted in the present study. However, it has to be noted that the incubation

time applied in the study of Rönnqvist et al. (2013) was significantly longer (incubation overnight) than the 45 min incubation time applied in this study. Remark also that in this study, as well as in the previous studies on swabbing mentioned in this paragraph, 'clean' surfaces are spiked and analyzed. In reality these surfaces will be contaminated with all sorts of residues which could affect the SRE. As such the SRE could be influenced negatively if the residue would firmly attach the viruses to the surface and hence protect the viruses from elution by vigorously swabbing. The presence of residues could also introduce inhibitors to the RNA extract and hence could introduce the necessity for analysis of higher dilutions of the initial RNA sample to circumvent possible inhibition and the occurrence of false negative results. However in this case the actual analyzed surface per PCR reaction would also decrease, and hence the sensitivity of the test would be lower. As such it is important during screenings to include an inhibition control.

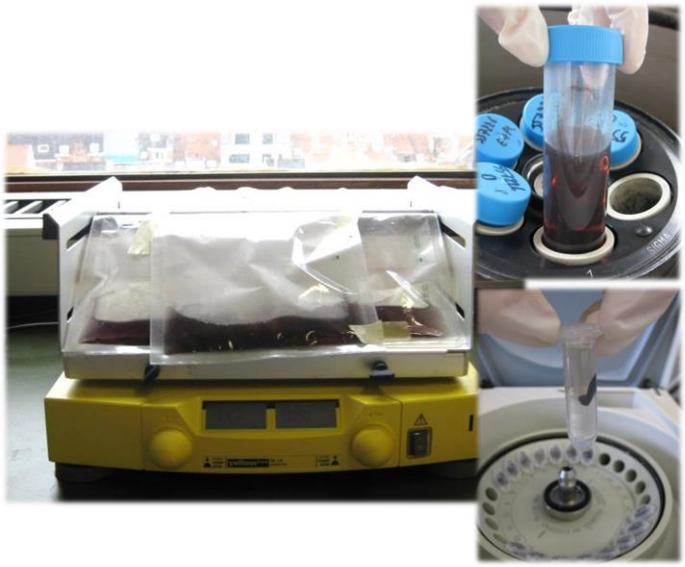
In conclusion, efficient swabbing continues to be a challenge due to the large variations in SRE, inherent to the swabbing process, and the possibility of substantial differences in SRE depending on the type of surface swabbed. The used biowipes (Biomérieux) in this study had the tendency to perform better than the commonly used cotton and polyester swabs, especially on coarser surfaces. In case of the use of normal cotton/polyester swabs, the semi-direct lysis method turned out to be more effective for the detection of NoV on larger surfaces than direct lysis (strategy 1), which is proposed by ISO/TS 15216-1. As such, one can question the efficiency of the direct lysis method proposed in the recently launched ISO/TS 15216-1:2012 for the detection of NoV on larger (100 cm^2) surfaces. The semi-direct lysis method has proven its effectiveness as well in this study as in the study of Rönnqvist, M. et al. (2013), for the detection of NoV on different types of surfaces.

CHAPTER 4

BATCH TESTING FOR NOROVIRUSES IN FROZEN RASPBERRIES

Redrafted from

De Keuckelaere, A., Li, D., Deliens, B., Stals, A., Uyttendaele, M. (2015).
Batch testing for noroviruses in frozen raspberries.
International Journal of Food Microbiology 192, 43-50.



Pictures of the elution-concentration protocol for the detection of NoV in raspberries

Authors contributions

Conception, planning, interpretation, and writing of the paper was done by Ann De Keuckelaere, Dan Li, Ambroos Stals, and Mieke Uyttendaele. The lab work and critical analysis was performed by Ann De Keuckelaere and Bart Deliens.

4. BATCH TESTING FOR NOROVIRUSES IN FROZEN RASPBERRIES

4.1. ABSTRACT

Berries, in particular raspberries, have been associated with multiple recalls due to norovirus contamination and were linked to a number of norovirus (NoV) outbreaks. In the present study a total of 130 samples of frozen raspberries were collected from 26 batches in four different raspberry processing companies. In two companies the samples consisted of bulk frozen raspberries serving as raw material for the production of raspberry puree (an intermediate food product in a business to business setting). In two other companies, the samples consisted of bulk individually quick frozen (IQF) raspberries serving as raw material for the production of frozen fruit mixes (as a final food product for consumer). Enumeration of *Escherichia coli* and coliforms was performed as well as real-time reverse transcription PCR (RT-qPCR) detection of GI and GII NoV (in 2 x 10 g). In addition, in cases where positive NoV GI or GII RT-qPCR signals were obtained, an attempt to sequence the amplicons was undertaken.

Six out of 70 samples taken from the 14 batches of frozen raspberries serving for raspberry puree production provided a NoV RT-qPCR signal confirmed by sequencing. Four of these six positive samples clustered in one batch whereas the other two positive samples clustered in another batch from the same company. All six positive samples showed NoV RT-qPCR signals above the limit of quantification of the RT-qPCR assay. These two positive batches of frozen raspberries can be classified as being of insufficient sanitary quality. The mean NoV level in 20 g of these raspberry samples was 4.3 log genomic copies NoV GI/ 20 g. The concern for public health is uncertain as NoV RT-qPCR detection is unable to discriminate between infectious and non-infectious virus particles. For the IQF raspberries, one batch out of 12 tested NoV positive, but only 1 out of the 5 samples analyzed in this batch showed a positive RT-qPCR GI NoV signal confirmed by sequencing. The RT-qPCR signal was below the limit of quantification of the assay used (< 3.7 log genomic copies/20 g). It was shown that the applied protocol for sequencing of the amplicon to confirm the specificity of the RT-qPCR signal was successful for GI NoV amplicons but often failed and provided an inconclusive result for GII NoV amplicons.

4.2. INTRODUCTION

Soft red fruits such as raspberries and strawberries have been repeatedly linked with foodborne outbreaks due to human norovirus (NoV) (Pönkä et al. 1999b; Mäde et al. 2013; Sarvikivi et al. 2012) and hepatitis A virus (HAV) (Reid and Robinson 1987; Nordic outbreak investigation team 2013). In the period 2009-2012 the EU Rapid Alert System for Food and Feed (RASFF) reported 10, 1 and 2 recalls/withdrawals due to the presence of NoV in frozen raspberries, frozen strawberries and other frozen (mixes of) berries, respectively. Within that same period (2009-2011) at least 28 outbreaks due to NoV contaminated raspberries and strawberries were identified in Europe (EFSA 2013). Furthermore in 2012 a huge outbreak affecting approximately 11,000 people occurred in Germany due to NoV contaminated strawberries (Mäde et al. 2013).

The increasing number of alerts and reported outbreaks has prompted national competent authorities and the fruit-based processing industry to establish a testing regime for NoV. The assessment of environmental conditions, good agricultural and hygienic practices in primary production and berry collection centers is important (FAO/WHO 2012) especially because subsequent steps in the production of frozen berries (or derived purees) may not be adequate to remove contamination. Nonetheless, it is well established that end product testing for food safety assurance has limitations, both due to the confidence one can have that the samples are representative of the batch, and also because methods of detection of pathogens, including the RT-qPCR methods for NoV are imperfect (ICMSF 2011). Nevertheless, this type of batch testing regimes is often applied in import controls or inspections by competent authorities (Ferrier and Buzby 2014). For example, as a consequence of the large 2012 NoV outbreak in Germany associated with frozen strawberries (Mäde et al., 2013), European Regulation (618/2013) was updated and mandated testing of imported frozen strawberries from China for NoV (n = 5, c =0, absence of NoV in 25 g). It is also common practice in supplier-buyer transactions, to monitor, by analysis, incoming raw material, or to provide a certificate of analysis per batch to its buyers. The intensity of such testing tends to increase when outbreaks or alerts occur related to specific commodities. This is presently the case for NoV in (frozen) raspberries. Although sampling plans are intrinsically limited in assessing the quality and safety of sampled foods, sampling might be useful to reveal major non-compliances and be a basis for analyzing performance trends so that corrective actions can be taken before loss of control (Lahou et al. 2014, ICMSF 2011). Detection of NoV genomic copies in frozen raspberries could be considered a useful parameter to be used for verification of good hygienic practice applied to berries at primary production and processing premises.

There are few reports of NoV screening in non-outbreak related samples. Maunula et al. (2013) sampled frozen raspberries, fresh raspberries and fresh strawberries at point of sale in three European countries (i.e. Czech Republic, Poland and Serbia). They reported that none of the 39 frozen raspberry samples (95% CI 0-9%), none of the 60 fresh raspberry samples (95% CI 0-6%), and none of the 21 fresh strawberry samples (95% CI 0-15%) were positive for NoV using RT-qPCR. Baert et al. (2011) reported 6.7% (10/150) of soft red fruits in a France study (unreported whether frozen or not) positive for NoV. Stals et al. (2011b) reported 4 of 10 raspberry samples (originating from Poland/Serbia) testing positive for NoV. These surveys are reporting single-sample subunit analysis, and there are currently no data available on NoV screening based upon a multiple-sample subunit approach (n=5) as is commonly used in setting microbiological standards.

RT-qPCR is currently the accepted standardized method for the detection of NoV in food including frozen berries, but is technically complex. The use of small analysis volumes of the nucleic acid extract produced after sample treatment, combined with the variable extraction efficiency, can result in the method being unable to detect virus below e.g. 10^2-10^3 genomic copies per sample. Also the production of the nucleic acid controls is also challenging and overall experience with implementation of RT-qPCR methods in surveys indicates that these methods might be further refined with regard to sampling, sample preparation, limit of detection and interpretation of results (EFSA BIOHAZ Panel 2014b, Stals et al. 2013b).

Overall, NoVs can be detected in frozen raspberries, but prevalence studies in particular using a multi-sample approach are limited, and quantitative data on viral load are scarce. Moreover, in many studies the focus has been on the technical elaboration of the NoV RT-qPCR detection method rather than the approach to interpretation of RT-qPCR results. The present study presents data on occurrence and levels of NoV in batches of frozen berries including the elaboration of a decision-tool for interpretation of NoV RT-qPCR and indicating batches of insufficient sanitary quality. In this study, sampling was performed on 26 batches (5 samples/batch) of frozen raspberries from four different frozen raspberry processing companies in Belgium. All batches originated from Poland, which is one of the biggest berry producing country in the EU. The outcome of the survey, limitations of this batch testing approach in assuring food safety and concerns of technical nature associated with RT-qPCR implementation and interpretation of results are discussed.

4.3. MATERIALS AND METHODS

4.3.1. Sampling plan

Sampling was selected to investigate the suitability of a multi-sample approach (n=5) and a decision-tool for interpretation of NoV RT-qPCR results on frozen berries, based on the resources available. A total of 130 convenience samples were collected from 26 batches in four different raspberry processing companies. In two companies (A and B) the incoming cooled and mixed/minced raspberries served as raw material for the production of raspberry puree (an intermediate food product). Samples were taken after initial storage of the incoming raspberries at -20°C at the processing site. In two other companies (C and D), the samples consisted of bulk individually quick frozen (IQF) raspberries serving as raw material for the production of frozen fruit mixes (as a final food product for consumers) or were intended for further distribution. IQF raspberries were sampled upon arrival in these companies. All 26 sampled batches, which originated from Poland, were picked in the summer of 2011 and 2012. From each batch, 5 samples of ca. 100 g raspberries were randomly taken by the companies themselves from different vessels/boxes of a batch. All samples were stored at -20°C in the lab and analyzed within two months. The total of 130 samples tested consisted of 14 batches of frozen bulk raspberries (n=70; 5 samples/batch) intended for the production of puree, and 12 batches (n=60; 5 samples/batch) of IQF raspberries.

From each sample, 20 g (2 x 10 g) was used for analysis of the presence of NoV using RT-qPCR detection as described below. Another 10 g was used for enumeration of *Escherichia coli* and coliforms by plating (pour plate method) a tenfold diluted suspension on RAPID' *E. coli 2* chromogenic agar medium (BioRad, France) and counting of typical colonies after 24h incubation at 37°C. The frozen raspberries were thawed overnight at 4°C before starting the NoV extraction procedure.

4.3.2. NoV RT-qPCR detection

4.3.2.1. Detection strategy

The detection strategy was adopted, with minor adjustments, from the one previously described by Stals et al. (2011b) and is schematically represented in Figure 4.1. In detail, the detection of NoV (in 20 g, 2 x 10 g subsamples) was performed according to the elution-concentration method described earlier (Stals et al., 2011c; Baert et al., 2008a). The first subsample (10 g) was spiked with 100 µl of an MNV-1 solution containing ca. 10^7 genomic copies of MNV-1 (first batch and second batch of aliquots contained initially 2.2 x 10^7 and 1.4 x 10^7 genomic copies of MNV-1 respectively) as a process control (PC). After spiking, the subsample with MNV-1 PC was incubated for 30 min at room

temperature before starting the virus extraction. The PC was used to calculate the recovery efficiency of the extraction process.

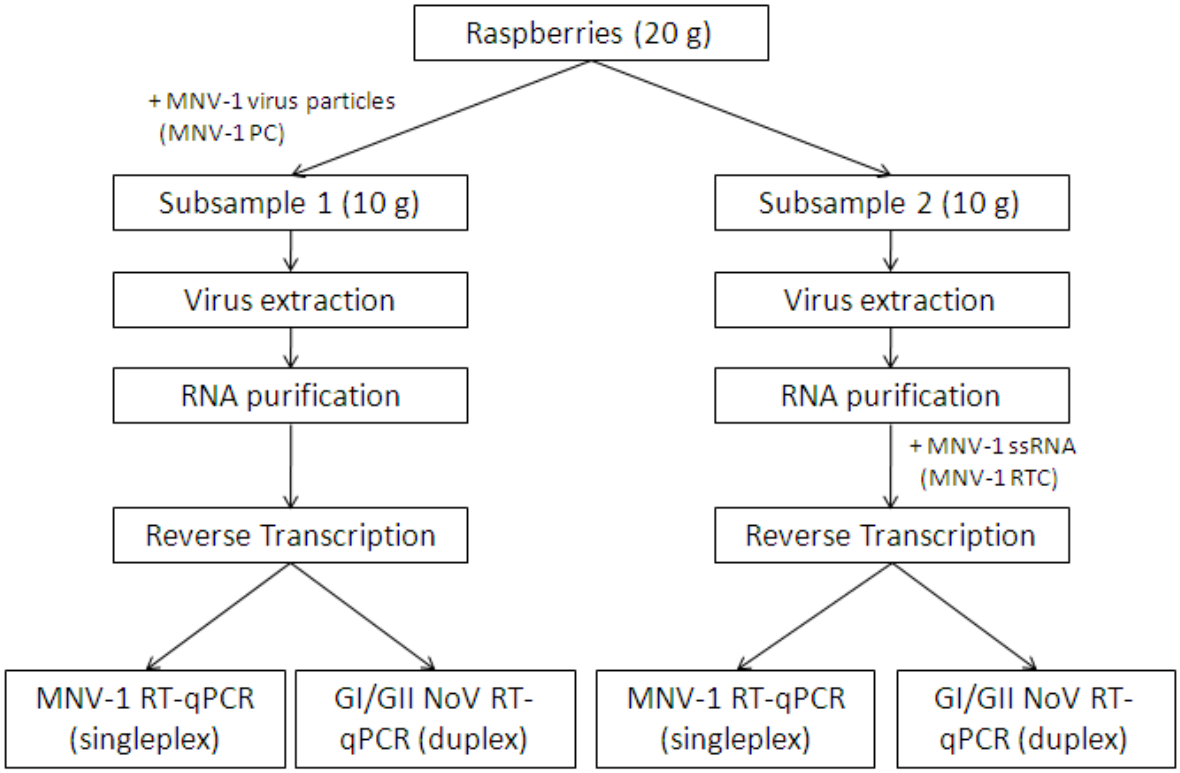


Figure 4.6. Overview of the detection strategy used for detection of NoV in raspberries. MNV-1: murine norovirus-1; NoV: norovirus; PC: process control; RTC: reverse transcription control; RT-qPCR: real-time reverse transcription PCR.

Virus extraction of the second subsample (10 g) was performed in parallel with the first subsample, but during the reverse transcription (RT) step 1 µl of MNV-1 genomic RNA (containing 10^3 to 10^4 copies) was added to the reaction mix as RT-control (MNV-1 RTC). No internal amplification control (MNV-1 IAC) was used during qPCR as previous results indicated that the RT-step is the most prone to inhibition and previous recovery efficiencies of the MNV-1 IAC were ca. 100% for raspberries (Stals et al. 2011b). The purpose of this MNV-1 RTC was to control the amplification efficiency and hence inhibition. For each subsample, qPCR was performed for the detection of MNV-1, either as PC or RTC. For each subsample, detection of GI and GII NoV was performed according to a duplex RT-qPCR as described earlier (Stals et al. 2009a).

4.3.2.2. Virus elution and concentration method

Virus extraction and elution using 30 ml tris/glycine/beef extract (TGBE) buffer (0.1 M Tris-HCl, 3% beef extract, 0.05 M glycine, pH 9.5 adjusted with 10 M NaOH) was performed as previously described by Baert et al. (2008a) and Stals et al. (2011c). To prevent gel formation, 150 µl of Pectinex (Sigma-Aldrich, Steinheim, Germany) was

added to the elution buffer. Afterwards, viruses were concentrated using the PEG 6000/NaCl precipitation technique. The final pellet was dissolved in 1.5 ml of phosphate-buffered saline (Lonza, Verviers, Belgium) of which 1 ml was subjected to further purification by the use of a chloroform-butanol purification step. The supernatant was stored at -80°C until further RNA purification.

4.3.2.3. RNA purification

Nucleic acid purification was performed using the automated NucliSens® EasyMAG™ system 2.0 (Biomérieux, Boxtel, the Netherlands) following generic 2.0.1 protocol for on-board lysis incubation according to manufacturer's guidelines. The final elution volume was 25 µl. All RNA isolations were stored at -80°C until further use.

4.3.2.4. Real-time reverse transcription PCR (RT-qPCR) for MNV-1, and NoV GI and GII detection

A two-step RT-qPCR was used for the detection of MNV-1 (PC/RTC), GI and GII NoV. The reverse transcription step was performed as described previously (Stals et al. 2011b) with the inclusion of 1 µl of MNV-1 RNA as a RTC in accordance to the detection strategy. In this protocol, 3 µl of a 1/10 dilution of the RNA purified from the virus extract was used in a total final volume of 20 µl. Subsequently, 5 µl cDNA was used for qPCR. All cDNA was stored at -20°C.

The qPCR was performed as described by Stals et al. (2009a), with the minor adaptation that MNV-1 detection was performed as a singleplex and GI and GII NoV were detected in a duplex reaction. Real-time quantification was performed on the SDS 7300 Real-time PCR System (Applied Biosystems). The used fluorophore/quencher combinations for GI/GII NoV and MNV-1 probes were 6-FAM/BHQ-1 (Integrated DNA Technologies), HEX/BHQ-2 (Integrated DNA Technologies) and FAM/MGBNFQ (Applied Biosystems), respectively. Each qPCR plate included two positive controls, containing fixed concentrations of both pGI and pGII plasmids diluted in either nuclease free water or diluted sample matrix, and at least eight no template controls (NTC) serving as negative control.

To establish the Limit Of Detection (LOD) and the Limit Of Quantification (LOQ) for all virus qPCR assays, and for subsequent quantification, a qPCR calibration/standard curve was established using the plasmids as described by Li et al. (2014b). For detection of MNV-1, standard dilution series of the control plasmid were included in each qPCR plate. Tenfold serial dilutions ranging from 10^7 to 10 copies of MNV-1 plasmids, analyzed in duplicate, were used to prepare the standard curves. The amplification data were collected and analyzed with the SDS 7300 instruments' software.

For GI and GII NoV detection, tenfold serial dilutions ranging from 10^6 to 10 copies of GI and GII NoV plasmids, in triplicate, were used as external standard curves. This series of plasmids were not included on the same 96 well reaction plates as the samples as a precautionary step to prevent false positive results due to cross-contamination (Stals et al., 2009b). Aliquots of the two positive controls, either diluted in nuclease free water or in diluted sample matrix, used during the qPCR detection of the samples were likewise included on the qPCR plate that contained the standard dilution series to control for the uniformity between the different qPCR runs. One single, optimal threshold for GI NoV detection and one for GII NoV detection was selected for all 5 qPCR runs (2 runs for mixed/minced raspberries, 2 runs for IQF and 1 run including standard dilution series for GI and GII detection). The amplification data was collected with the SDS 7300 instruments' software but analysis (quantification) was done using Microsoft Excel 2010 (version 2007).

4.3.2.5. Confirmation of specificity of NoV GI and GII RT-qPCR amplicons by sequencing

The plasmids for GI (pGI) and GII NoV (pGII) (described by Li et al. 2014b) used in this study included a contamination marker 'GGATCC' sequence in the amplicon region between primer and probe binding sites. This sequence is not present in amplicons derived from genuine positives for GI and GII NoV. As such, positive RT-qPCR results could be confirmed by sequencing the amplicons to exclude false positives due to cross-contamination with the two positive control samples (containing GI and GII plasmids) present on each plate. Direct sequencing using the same forward and reverse primer as used during the qPCR step was not an option due to the small amplicon size and the difficulty degenerated primers pose on sequencing without cloning cDNA fragments (Williams-Woods et al. 2011). In order to overcome these obstacles, qPCR amplicons of interest were re-amplified by conventional PCR using NoV GI and GII primers that were tailed on the 5'-end with the M13 forward (CAGGAAACAGCTATGACC) and reverse sequence (TGTAAAACGACGGCCAGT) as previously proposed by Williams-Woods et al. (2011). This modification added an additional 36 bp to the original amplicon size allowing direct sequencing of the amplicons using complementary M13 primers. The protocol used for this PCR was: 95°C for 8 min, followed by 30 cycles of 94°C for 30 s, 60°C for 1 min, and 75°C for 30 s, with a hold of 72°C for 10 min at the end. After extension, PCR amplicons were detected by gel electrophoresis using 2% agarose gels and visualized using ethidium bromide to control whether only one singular band of the correct size was visible. When this was confirmed, the PCR product was sequenced in both directions using M13 primers by Macrogen (Amsterdam, the Netherlands).

4.3.3. Data analysis

4.3.3.1. Interpretation of NoV GI and GII RT-qPCR signals

Each qPCR run was validated by the negative and positive control samples included in each run. A sample of frozen raspberries was determined to be NoV positive if each of the three following requirements was met:

(i) A NoV RT-qPCR signal was accepted as a positive signal if the Ct-value obtained was below the Ct of the *Limit Of Detection* (LOD) ($Ct \leq$ Ct LOD) as established from the calibration curve or if the Ct-value was below the cut-off value of 40.0 if the latter case was more restrictive (when Ct LOD > 40.0). Samples with a Ct-value higher than 40 or higher than the established Ct LOD (when Ct LOD $\leq$40.0) were deemed to be RT-qPCR background signals and thus judged as a negative test result for the NoV RT-qPCR test (Bustin et al., 2009).

(ii) The NoV RT-qPCR amplicons were confirmed to match the targeted NoV GI or NoV GII region by sequencing.

(iii) At least one out of two frozen raspberry 10 g subsamples showed a positive NoV RT-qPCR signal (either for NoV GI RT-qPCR or for NoV GII RT-qPCR or for both). The positivity rate (1/2 or 2/2) represents the fraction of positive subsamples of 10 g.

4.3.3.2. MNV-1 controls

To control for false-negatives due to a failed extraction or due to inhibition of the molecular detection assay, a process control (PC) and a reverse transcription control (RTC) were included for each sample. Both controls consisted of a dilution of MNV-1 lysate and diluted, purified MNV-1 ssRNA, respectively. The recovery efficiency of the spiked MNV-1 virus (used as PC or RTC) from an individual sample was calculated using the following equation: recovery efficiency (%) = the number of recovered MNV-1 genomic copies / the number of seeded MNV-1 genomic copies x 100. The recovery success rate for any specific matrix type of raspberries for the detection of MNV-1 PC and MNV-1 RTC was calculated using the following equation: recovery success rate = number of 10 g analyses in which detection of MNV-1 PC or MNV-1 RTC was possible/number of performed analyses.

4.4. RESULTS

4.4.1. Bacteriological quality of frozen raspberries

All 130 samples tested negative for the hygiene indicators *E. coli* and coliforms (<10 CFU/g) and thus all 26 tested batches of raspberries were considered to be of good bacteriological sanitary quality.

4.4.2. Parameters of the NoV molecular detection strategy

The linear dynamic range for GI and GII NoV quantitative detection ranged from 10^6 to 10 genomic copies (GC). Table 4.1. contains the parameters of both standard curves. The Ct-value corresponding to this lower limit of 10 GC being defined as the Limit Of Quantification (LOQ) was 37.5 and 35.7 for GI and GII NoV qPCR, respectively. The theoretical Limit Of Detection (LOD) (corresponding to the detection of 1 GC by extrapolation) was Ct-value of 40.7 and 39.3 for GI and GII NoV qPCR, respectively. Thus when the Ct-value of a samples was between 37.5 and 40.0 or between 35.7 and 39.3 for the detection of GI and GII NoV respectively, NoV GC were deemed to be present, but not quantifiable ('+' in Table 4.2.). When the Ct-value of a sample was higher than 40.0 or >39.3 for detection of GI and GII NoV respectively, a negative test result was assigned for the NoV RT-qPCR test ('-' in Table 4.2.). When the Ct-value was ≤37.5 or below 35.7 for the detection of GI and GII NoV respectively, the positive RT-qPCR result was considered to be quantifiable and was indicated with 'log of genomic copies detected' in Table 4.2. and the quantity derived from the standard curve. When both subsamples (each 10 g) were NoV positive and quantifiable, GC detected by RT-qPCR were added together.

Table 4.3. Data obtained from the control plasmid standard curves of the real-time RT-PCR performed for the quantification of GI and GII NoV.

Parameters[a]	GI NoV RT-qPCR	GII NoV RT-qPCR
Slope	-3.76	-3.56
E_a [b]	84.43%	90.77%
Y-intercept when X = 0.0	40.7	39.3
R^2 [c]	1.00	1.00
LOD	40.7[d]	39.3
LOQ	37.5	35.7

[a] Control plasmids were used as standard, as such viral loadings were expressed in number of genomic copies detected; the tenfold dilution series were analyzed in triplicate

[b] The amplification efficiency of the real-time RT-PCR was calculated according to the formula: $E_a=(10^{-1/slope})-1$, where $E=1$ corresponds to 100 % efficiency

[c] The correlation coefficient

[d] As the theoretical LOD >40.0, the Ct-value of 40.0 was set as a more restrictive cut-off level for the presence of GI NoV.

The quantities of GC of GI or GII NoV found in an individual sample (20 g) are calculated by multiplying the GC detected in 5 µl cDNA by qPCR with the dilution factor (x 500 = 3/2 x 250/3 x 4/1) which takes into account the fractional use of the virus concentrate (2/3), the purified RNA (3/250) and the cDNA (1/4). As such, the theoretical detection limits in the event of positive GI and GII NoV RT-qPCR signals were 765 (2.9 log) NoV GC/10 g (Ct-value = LOD = 40.0) and 500 (2.7 log) NoV GC/10 g (Ct-value = LOD = 39.3), respectively. The theoretical limit of quantification (tLOQ) is 5000 (3.7 log) NoV GC/10 g.

4.4.3. NoV detection in frozen raspberries

The results of NoV GI and GII RT-qPCR detection in frozen raspberries are summarized in Table 4.2. Six of 70 raspberry samples intended for the production of raspberry puree showed presence of NoV RNA. The presence of NoV RNA means according to our definition in the present study the presence of by sequencing confirmed RT-qPCR positive signals for either GI NoV or GII NoV or both GI and GII NoV in at least one out of the two 10 g subsamples. As such, all six NoV RNA positive samples showed at least for one of the two subsamples a NoV GI RT-qPCR signal. All positive GI RT-qPCR signals had a Ct-value below LOQ, and were confirmed to be NoV RNA by sequencing. It is noted that in four samples only one of the two 10 g subsamples was positive for NoV GI by RT-qPCR. When one sample of a batch was confirmed positive for NoV RNA, the whole batch was considered positive. The mean viral load (per 20 g mixed/minced raspberries) of these six samples was 4.3 log NoV GI genomic copies. Five of these six samples also showed NoV GII RT-qPCR positive signals (Ct-value <LOD). All, except one of these GII RT-qPCR positive sample, had a Ct-value ranging between LOD and LOQ (as such not quantifiable and indicated with a '+' in Table 4.2.) and a positivity rate of 1 out 2. However, the sequencing results of these assumed NoV GII PCR amplicons, were inconclusive for four out of five samples. In these four samples, sequencing resulted only in the sequence of the extended primers, despite the successful sequencing of the extended amplicons of the GII NoV plasmid controls. These four NoV GII RT-qPCR positive samples were thus classified as non-confirmed for presence of NoV GII RNA (indicated with a star in Table 4.2.). But as these four samples already showed a confirmed NoV GI RT-qPCR signal, they were overall already attributed as being positive for presence of NoV RNA according to the definition outlined earlier (section 4.3.3.1.). For one sample, sequencing of the NoV GII PCR amplicon was successful and thus a confirmed presence of NoV GII RNA (although not quantifiable, '+') was noted. This one sample was also already confirmed to contain NoV GI RNA (4.9 log genomic copies). Overall the results indicating presence of NoV RNA in the raspberry puree samples, indicated that all six of

these NoV RNA positive samples were clustered in two batches from Company A. Four out of five samples in batch 1 and two out of five samples in batch 2 contained NoV RNA.

For IQF raspberry samples, initially 6 samples showed NoV RT-qPCR signals indicating either possible presence of GI NoV and/or GII NoV. After sequencing, only one sample was confirmed to contain NoV GI RNA and two samples, with NoV GII RT-qPCR signals, did not give conclusive results (marked with '*'). Therefore, these inconclusive, positive RT-qPCR signals could be either due to the presence of NoV RNA or the presence of 'positive control plasmid DNA'. The three other samples with NoV RT-qPCR signals, one subsample with a signal for GII NoV and two subsamples with signals for possible GI and GII NoV presence, were confirmed to contain positive control plasmid DNA, and were classified as not containing NoV RNA (indicated with '-' as negative). In conclusion at least one out of 12 batches of IQF raspberries contained NoV RNA, and possibly two other batches. However, quantities of NoV were below the limit of quantification (< 3.7 log genomic copies GI or GII NoV/20 g) in all (potential) positive NoV RNA samples.

Table 4.2. Detection of GI and GII NoV RNA in 26 batches of raspberries. In company A and B mixed/minced raspberries were sampled that were intended for the production of puree, and in company C and D IQF raspberries were sampled.

Company	Batch n°/Sample n° (B n°/S n°)	NoV presence/ absence	Log genomic copies of NoV GI in 20 g raspberry (Ct values) [positivity rate]			Log genomic copies of NoV GII in 20 g raspberry (Ct values) [positivity rate]		
A	B1/S1	**P**	5.1	(31.88;34.90)	[2/2]	4.2*	(33.75;36.18)	[2/2]
	B1/S2	**P**	4.2	(35.13)	[1/2]	+*	(36.82)	[1/2]
	B1/S3	**P**	4.9	(34.70;33.07)	[2/2]	+	(36.82)	[1/2]
	B1/S4	**P**	4.5	(34.01)	[1/2]	+*	(36.39)	[1/2]
	B1/S5	**A**	-			-		
	B2/S2	**P**	3.7	(36.94)	[1/2]	-		
	B2/S3	**P**	3.7	(36.95)	[1/2]	+*	(37.5)	[1/2]
	B2/S1,4&5	**A**	-			-		
	B3-> B7	**A**	-			-		
B	B1->B7	**A**	-			-		
Total puree		**2/14**	batches positive for NoV RNA (confirmed by sequencing)					
C	B1&B2	**A**	-			-		
	B3/S 1,3->5	**A**	-			-		
	B3/S2	**P***	-			+*	(37.33)	[1/2]
	B4/S1->5	**A**	-			-		
	B5/S1,2,4,5	**A**	-			-		
	B5/S3	**P**	+	(38.00)	[1/2]	-		
	B6/S1->5	**A**	-			-		
	B7/S1,2,4,5	**A**	-			-		
	B7/S3	**P***	-			**+***	(38.24)	[1/2]
D	B1->B5	**A**	-			-		
Total IQF	Minimal	**1/12**	batches positive for NoV (confirmed by sequencing)					
	Maximal	**3/12**	batches positive for NoV (however of two batches the positive sample was not confirmed by sequencing)					

'-': no NoV detected (# < LOD)
'+': defined as positive for NoV: positive RT-qPCR signal (amplification curve) + confirmation by sequencing of amplicon
'*': Positive RT-qPCR signal (amplification curve), but inconclusive result by sequencing.

4.4.4. MNV-1 controls

Only the 1/10 diluted RNA was used for RT-qPCR detection of both MNV-1 controls and human NoV as preliminary experience with these raspberry food matrices indicated that, despite the inclusion of a chloroform-butanol purification step, there was a considerable degree of inhibition (data not shown) of the RT-step and/or the qPCR-step.

Results for the efficacy of the recovery of the MNV-1 PC and the MNV-1 RTC are summarized in Table 4.3. The recovery efficiencies of MNV-RTC, which was used as a measure for the amplification efficiency and hence inhibition, were significantly lower for detection in mixed/minced raspberries intended for puree (40.2% ± 16.6%) than those obtained for detection in IQF raspberries (76.0% ± 25.7%) (t-test, p= 0.000). The mean recovery efficiency for the detection of MNV-1 PC in both types of raspberries was 4.7% ± 4.2% and 10.1% ± 12.4% for mixed/minced raspberries and IQF raspberries, respectively. However, a significant higher mean recovery efficiency of the PC (28.7% ± 7.0%) was noted for the three batches of IQF raspberries analyzed in the last sampling week compared to the mean recovery efficiency of the previous 9 batches of IQF raspberries (2.4% ± 2.6%) analyzed in the first 3 sampling weeks (t-test, p = 0.000). This rise in recovery efficiency coincided with a shift in the batch of aliquots of diluted MNV-1 being used as PC to spike one of the duplicate 10 g subsamples upon analysis. Although both batches of PC were derived by 1/10 dilution from the same stock of 1/10 diluted lysate of MNV-1, and were verified to contain approximately the same number of genomic copies when the initial stock was tested (ca. 10^7 genomic copies/100 µl), the recovery efficiencies were significantly higher when the latter stock was used.

Table 4.3. Quantitative and qualitative analysis of the extraction efficiency (MNV-1 PC) and amplification efficiency (MNV-1 RTC).

Terminology of level of recovery efficiency		Fraction of samples with recovery efficiency between the specified boundaries			
		MNV-1 PC		MNV-1 RTC	
		Raspberry (puree)	Raspberry (IQF)	Raspberry (puree)	Raspberry (IQF)
Very low	0.1% ≤ % < 1%	0.07	0.23	0.00	0.00
Low	1% ≤ % < 10%	0.81	0.48	0.00	0.00
Moderate	10% ≤ % < 25%	0.12	0.10	0.13	0.00
High	25% ≤ % < 70%	0.00	0.18	0.79	0.47
Very high	≥ 70%	0.00	0.00	0.09	0.53
% mean recovery efficiency ± SD		4.7%±4.2%	10.1±12.4%	40.2%±16.6%	76.0%±25.7%
Recovery success rate		70/70	60/60	70/70	60/60

4.5. DISCUSSION

It is well acknowledged that there are limitations of testing and batch control for ensuring food safety. This primarily relates to the restriction on the units that can be sampled from a batch. Apart from statistical limitations of sampling plans, estimates from the performance of sampling plans do not take into account errors that might occur from the microbiological methods used to determine the presence of the pathogens under considerations in these foods (ICMSF 2011). As an example of shortcomings of batch testing to evaluate food safety: if the sample sub-population of a batch is e.g. 100, 20, 10 or 5 units, and in none of the samples the pathogen is detected, it can be estimated (with 95% probability) that the maximum number of units that is contaminated is respectively less than 3%, 14% 26% and 45% of the tested batch. Thus the tested sample units may not contain the pathogen under consideration even when the batch is contaminated. For example, during analysis of the batch of frozen strawberries (44 ton) implicated in the German outbreak of NoV in 2012, NoV genomic copies could not be detected in 4/11 tested samples, which could be the result of inadequate testing sensitivity or heterogeneous NoV contamination (Mäde et al. 2013).

The sampling plan in the present study targeted frozen raspberries (either IQF or frozen for further processing to puree) from Poland. Poland is the most important producer of berries for further processing (freezing) within EU, a main supplier of frozen berries to the four processing companies who collaborated in this study and frozen berries from Poland have been implicated in EU RASFF alerts previously. NoV analysis is a laborious and time-consuming analysis, restricting the number of samples that could be performed in this multi-sample survey. Initially, 28 batches were targeted (7 per company), knowing that at the sampling plan frequency, even when all batches tested negative, a contamination level up to 10% could have been present. Eventually, 26 batches (2 batches less due to some communication issues on sampling with some companies) were surveyed and quantifiable NoV RT-qPCR signals were detected in two batches (of mixed berries for further processing to puree) having multiple samples positive. This indicates 8% (2/26; 95% CI 2-24%) can be classified as being of insufficient sanitary quality and in need of corrective measures. Furthermore another batch of IQF raspberries showed single sample result, with RT-qPCR high Ct value, thus not quantifiable but detected.

In the majority of 20 g samples showing positive for NoV RNA, only 1 out of the two replicate samples were positive (positivity rate = 1/2). However, this negative replicate sample needs not necessarily to be judged as a false negative result. Positive NoV RT-qPCR signals are often associated with high Ct values, in the range of Ct 35 to 38, close to the limit of detection. As defined, the limit of detection is the smallest concentration of the

analyte that can be detected in a sample with a given probability (e.g. 50% or 1 out of 2) (Anonymous 2013). The sensitivity of a detection method is usually described as the fraction of the total number of positive cultures correctly assigned and low sensitivity of a detection method will lead to false negative results. The issue in the present survey of naturally contaminated frozen raspberries is that the "true" result and hence prevalence is unknown. Therefore it is difficult to interpret whether a negative result of a replicate result (the 1 out of 2), or the negative result of another sample of the same batch is to be judged as a "false negative" or is "true negative". Also intra-batch heterogenity in NoV contamination may occur and thus analyzing another 10 g in the replicate sample or sampled at another location in the batch may indeed lead to a similar or different result.

Although the number of samples/batches analyzed is limited, the results enabled us to discuss on sampling, on interpretation of qPCR-generated results for viruses and to make a contribution to these knowledge gaps. This study reports the analysis of NoV in frozen raspberries using established standard RT-qPCR methods. The presence of "plasmid control sequence" was detected in a total of three sample wells in the two qPCR plates used for the detection of GI and GII NoV in IQF raspberry samples. Therefore, it can be concluded that cross-contamination took place, even though all negative template controls (in total 27 NTC's) and all blank control samples implemented during RNA extraction (7 controls) and RT-PCR (6 controls), which were included on the same two qPCR plates, had negative NoV RT-qPCR signals. These false positive results can be attributed to contamination with plasmid DNA that was present on each plate as a positive control. This highlights that even under stringent control conditions to prevent contamination, and even though all negative controls included in the experimental set-up were negative, seemingly this type of carry-over contamination can still occur. However, in this study this problem was only revealed because of the efforts made to confirm results by sequencing. The confirmation of NoV RT-qPCR results is still challenging and not a common procedure in most of the laboratories investigating NoV contamination in foods.

The RT-qPCR detection strategy for NoV in food used in this study included a process control (MNV-1 PC) and an external control RNA (MNV-1 RTC) to assess the extraction efficiency and the amplification efficiency/inhibition, respectively. It is assumed that a minimum amplification efficiency for the recovery of the external control RNA (MNV-1 RTC) of >25% is sufficient (ISO/TS 15216-1:2012(E)) (Anonymous 2012c). This threshold was met for all IQF raspberry samples, but 13% of mixed/minced raspberry samples intended for puree had an amplification efficiency in the range 12% - 24%. The extraction efficiencies (recovery efficiency of MNV-1 PC) were categorized as low to high, with at least >75% of analyses resulting in a recovery efficiency of ≥1%. With a

fraction of samples having a recovery efficiency (MNV-1 PC) <1%. This is not unusual, as Mattison et al. (2010) found 17% of samples, analyzed for enteric viruses in leafy greens, had a process control recovery below 0.01%. The recovery efficiencies obtained in this study for both MNV-1 controls are comparable with those obtained in the Belgian (Stals et al. 2011b) and French study (Baert et al. 2011) for the detection of NoV in soft red fruits.

However, direct comparison of the process control recovery between studies is not always relevant, as other factors besides the used protocol or type of control virus can influence the obtained recovery efficiency. Nevertheless, the authors support the use of a PC in order to benchmark the obtained extraction efficiencies. Furthermore, the production of process controls with consistent quality is challenging. For example here a significant difference was observed in the recovery efficiency of MNV-1 PC, when a second, freshly prepared batch of aliquots derived from the same initial 1/10 diluted stock of MNV-1 lysate was used for the analysis of the remaining IQF raspberry samples. The use of different stocks of MNV-1 PC was expected to be the reason for the difference in recovery efficiencies. As all other possible relevant parameters that could have influenced the recovery efficiency (e.g. reagents and analysts) were kept constant and all batches of IQF raspberries were processed sequentially, with limited time intervals (maximum one week). A hypothesis for the reason for the higher recovery efficiency obtained when the second, more freshly made batch of PC control was used, could be a difference in the ratio of infective viruses/genomic copies. Differences in the yield of viral concentration methods for non-infectious particles (genomic RNA) versus infectious particles have been reported (Gassilloud et al. 2003), as some viral concentration methods can be based on the particle structure of the viruses (Haramoto et al. 2007) and hence favor the detection of RNA that is encapsulated (possibly infective) over free RNA. Meanwhile the differences in the recovery efficiency of MNV-1 batches did not seem to be relevant for the finding of NoV RNA positive samples, as all analyzed raspberry samples processed during the last sampling week, using the newer batch of aliquots of PCs, showed negative for NoV RNA. Whereas when quantifiable NoV RT-qPCR results were obtained in the present study this occurred when the first stock of MNV-1 was used (the latter observed to lead to a lower recovery efficiency).

Experiences obtained using process controls in large screening studies is not abundant as not all screening studies that used a sample process control and/or inhibition control, specify the obtained recovery efficiencies (e.g. Kokkinos et al. 2012; Maunula et al. 2013) and other studies have not included any process/inhibition control (e.g. Serracca et al. 2012; Yilmaz et al. 2011). Clearly, standard inclusion of the extraction recoveries obtained would provide valuable information on the use of a PC and could potentially reveal

experienced variabilities and difficulties when using the current protocols and surrogate viruses. Hence the authors support the standard inclusion of a PC and encourage the reporting of these results. To verify our hypothesis and to obtain a standardized process control procedure, it would be of interest to study the effect the process control quality has on the obtained recovery efficiency in more detail. This in order to formulate a best practice scenario to standardize this PC batch quality and hence enabling better benchmarking of the resulting recovery efficiency. This could help improve the availability of reliable quality control materials produced independently. A recent EFSA opinion on NoV in leafy greens suggested this as a necessary prerequisite before there can be complete confidence in the concordance of results within and between laboratories (EFSA BIOHAZ Panel 2014b).

In the present study, natural occurrence of NoV in processed raspberries (not involved in any outbreaks) was demonstrated in three batches, even though all batches tested negative for the bacterial hygiene indicators *E. coli* and coliforms. The two positive mixed/minced raspberry batches each from company A were destined for further processing into puree. In this puree production process or during further processing to consumer end products, a heat treatment up to 90°C is generally included. As such, the resulting level of concern for public health raised by the end product (puree after heat treatment) of both batches is considered to be low, as cooking procedures in which the internal temperature of the food reaches at least 90°C for 90 s are considered adequate treatments to destroy viral infectivity in most foods (Anonymous 2012d; FAO/WHO 2012). For company C (IQF products), the one positive batch for GI NoV RNA needs further action. Since the finding of NoV RNA can be considered as the result of viral, fecal contamination and hence could indicate possible sanitary problems in the farm-to-fork chain. As such, the finding of NoV genomic copies, however incidental and below LOQ, may provoke corrective actions and verification of on-farm agricultural and hygienic practices. However, no quantifiable results for the presence of NoV genomic copies were detected (only Ct values between LOD and LOQ), and the obtained Ct-value was close to the limits of the qPCR detection method. The two presumptive positive batches for GII NoV (inconclusive results by sequencing) are considered as negative according to the decision tree elaborated in §4.3.3.1. However could not be classified as ‘true negative’ due to inconclusive results by sequencing and hence should be reanalyzed.

In conclusion, the current study presents data on the occurrence of NoV in frozen raspberries (mixes or IQF). The presence of quantifiable amounts of NoV and the clustering of several positive samples in one batch can indicate sanitary problems, although the impact to public health is unknown (Stals et al. 2013b). The identification of false

positive batches, even though all implemented controls were negative, demonstrated the added value of additional confirmation of positive RT-qPCR signals. As also in a situation of adherence to good laboratory practices, contamination can occur randomly and hence cannot be excluded (Stals et al. 2009b) or necessarily detected by the use of negative controls. Specificity and confirmation of the RT-qPCR signal and resultant amplicon was demonstrated successfully. However, sequencing often failed and consequently gave inconclusive results for GII NoV RT-qPCR signals.

The present study demonstrated a defined approach for the interpretation of NoV-RT-qPCR signals obtained and puts this in a context of batch sampling, considering five units per batch as typically applied in sampling plans for import control or inspections to assess the safety of frozen raspberries. In only one batch consistent quantifiable NoV genomic copies were obtained in 4 out of the 5 sampled units. Most only single unit positive NoV RT-PCR signals were obtained in a batch. Although testing of batches for supplier control and import control is an option, it remains the question whether this is cost-effective and the most appropriate manner to guarantee food safety. As stated by CAC/GL 79-2012, in the application of general principles of food hygiene to the control of viruses in food, effective control strategies need to focus on prevention of contamination. For soft red fruits this means that extra care must be taken at pre-harvest, harvest level and at the post-harvest phase, in order to prevent contamination due to inappropriate (irrigation) water and fertilizer or due to contact with infected food handlers.

CHAPTER 5

QUANTITATIVE STUDY OF CROSS-CONTAMINATION WITH *E. COLI*, *E. COLI* O157, MS2 PHAGE AND MURINE NOROVIRUS IN A SIMULATED FRESH-CUT LETTUCE WASH PROCESS

Redrafted from

Holvoet, K.*, De Keuckelaere, A.*, Sampers, I., Van Haute, S., Stals, A., Uyttendaele, M. (2014). Quantitative study of cross-contamination with *Escherichia coli*, *E. coli* O157, MS2 phage and murine norovirus in a simulated fresh-cut lettuce wash process. Food Control 37, 218-227.

* These authors contributed equally to this work

Authors contributions

Conception and planning of the paper was done by Kevin Holvoet, Imca Sampers, and Mieke Uyttendaele. Critical analysis and interpretation of results concerning *E. coli* was performed by Kevin Holvoet, Sam Van Haute, Imca Sampers, and Mieke Uyttendaele. Critical analysis and interpretation of results concerning MS2, MNV-1, and *E. coli* O157 was performed by Ann De Keuckelaere, Ambroos Stals, and Mieke Uyttendaele. Lab work concerning *E. coli* was done by Kevin Holvoet and lab technicians. Lab work concerning MS2, MNV-1, and *E. coli* O157 was done by Ann De Keuckelaere, Ambroos Stals, and dedicated lab technicians. Writing was coordinated by Kevin Holvoet and Ann De Keuckelaere with input of all other co-authors.

5. QUANTITATIVE STUDY OF CROSS-CONTAMINATION WITH *E. COLI*, *E. COLI* O157, MS2 PHAGE, AND MURINE NOROVIRUS IN A SIMULATED FRESH-CUT LETTUCE WASH PROCESS

5.1. ABSTRACT

The water management in fresh-cut produce processing is an important factor affecting the microbial quality and safety of fresh-cut produce. For this chapter, a commercial lettuce washing process was simulated using two subsequent washing baths (WB). The worst case scenario, when no sanitizers are used and still common in some European countries, was investigated to fully understand the potential for cross-contamination and to obtain baseline data for further risk assessment. The two cross-contamination processes (from lettuce to water and from water to lettuce) were included in the simulation study, and the transfer of *E. coli*, *E. coli* O157, MS2 phage and murine norovirus was quantified. The mean reduction of initially contaminated lettuce through the washing in two successive WB was limited: 0.3 $\pm$ 0.1 log CFU *E. coli*/g after washing in WB1 and an additional reduction of 0.2 $\pm$ 0.1 log after WB2. The microbial load of the water in the washing baths, initially starting off with potable water, increased rapidly during the washing process of the contaminated lettuce. Furthermore to quantify the transfer of the four implicated micro-organisms from contaminated water to the lettuce, the first washing bath was inoculated with either approximately 3.0, 4.0 and 5.0 log CFU *E. coli*/100 ml or 4.8, 5.6 or 6.7 log CFU *E. coli* O157/100 ml, 4.0, 5.1 or 6.5 log PFU MS2 phages/100 ml or 6.5 log PFU/100 ml norovirus surrogate MNV-1. The contamination of the subsequently washed lettuce portions resulted in levels of ca. 1.0 up to 1.9 log CFU *E. coli*/g after passing the two washing steps. In addition, after a rapid initial increase, due to spill over of water from WB 1 to WB2, the contamination of WB2 further augmented during the washing process to approximately 1.0 to 0.5 log below the inoculation level of WB1. Transfer of *E. coli* O157, MS2 phages or MNV-1 from the water to the lettuce was respectively 0.9% ± 0.3%, 0.5% ± 0.2% and 0.5 ± 0.1% after WB1 and resulted in a contamination level for the highest inoculum level of WB1 of respectively ca. 2.9 ± 0.1 log CFU/g, 3.7 ± 0.1 log PFU/g and 4.4 ± 0.1 log PFU/g lettuce. The quantitative data of lettuce contamination and transfers established in this chapter further highlight the vulnerability of fresh produce to cross-contamination during the washing stage. It stresses that notwithstanding the use of initial potable water and partial refreshment of water but without the use of sanitizers, microbial pathogens (or indicator organisms) may easily be introduced and reside for prolonged periods in washing baths enabling cross-contamination to the final fresh-cut product.

5.2. INTRODUCTION

Recently, a number of outbreaks have been associated with fresh-cut vegetables emphasizing the vulnerability of fresh-cut processing (CDC 2010; Fisher and O'Brien 2001, Friesema et al. 2008; MacDonald et al. 2011). The processing line of commercial fresh-cut vegetables consists of a succession of different unit operations, including storage of the produce, trimming, cutting/slicing/shredding, washing, draining, rinsing, centrifugation and packaging (Allende et al. 2004; Baur et al. 2005; Delaquis et al. 2004). Ready-to-eat produce is not subjected to any microbial inactivation such as surface pasteurization or cooking, which makes leafy greens an important transmission route for foodborne bacteria (Friesema et al. 2007; Horby et al. 2003; Wendel et al. 2009) and viruses (Ethelberg et al. 2010; Oogane et al. 2008). The current control measures rely on washing and cooling below 4 °C to restrict the microbial load and growth (Bhagwat 2006; Lehto et al. 2011; Luo 2007). Washing of produce removes soil, plant debris, pesticide residues and cell exudates that support microbial growth (Baur et al. 2004; Gil et al. 2009). It reduces produce temperature to suppress physiological disorders and microbial growth during subsequent storage (Delaquis et al. 2004; Gil et al. 2009). However, low temperatures may contribute to the persistence and subsequent transmission of viruses to the human host (Seymour and Appleton 2001). Though water is a useful tool for reducing potential contamination, several publications both in real life and lab-scale showed that washing water of inadequate quality has the potential to be a direct source of contamination and a vehicle for spreading localized bacterial and viral contamination (Allende et al. 2008; Baert et al. 2009b; Gerba 2009; Gil et al. 2009; Holvoet et al. 2012; Luo 2007; SCF 2002; Seymour and Appleton 2001;Wachtel and Charkowski 2002). As antimicrobial agents in fresh-cut produce washing such as chlorine are prohibited in several European countries such as Germany, the Netherlands, Denmark and Belgium (Artes and Allende 2005; Artes et al. 2009; Rico et al. 2007), fresh produce processing relies on continuous addition and refreshments of washing baths with large amounts of potable water, up to 40 m^3/ton of raw produce, to minimize the event of accumulation of micro-organisms in the water and transfer of micro-organisms from the water to the fresh-cut lettuce (Olmez and Kretzschmar 2009; Selma et al. 2008; VMM 2005).

The two cross-contamination events (from lettuce to water and from water to lettuce) were performed separately to gain more insight in transfers and degree of contamination established. Bacterial (cross-) contamination with *Escherichia coli* (O157) was investigated as *E. coli* is a well-known indicator for fecal contamination and the possibility of the presence of other more harmful organisms such as pathogenic *E. coli*, *Campylobacter* spp., *Salmonella* spp. (Wilkes et al. 2009). Firstly, inoculated lettuce was used in the simulation to show the impact of a contaminated incoming raw product on the

wash water quality of the two washing baths. Secondly, non-inoculated lettuce was washed in simulated "reconditioned used" water (water that was used for washing of multiple portions of lettuce in a row) of different microbial qualities to determine the degree of cross-contamination between water and lettuce. Finally, the influence of a final potable water rinsing step after the washing steps was evaluated with respect to the effect on the microbial load.

Viral foodborne outbreaks related to leafy greens such as lettuce have been reported frequently (Dewaal and Bhuiya 2009; EFSA 2013). Viral transfer from contaminated fresh produce to the washing water has been documented in several disinfection studies (Baert et al. 2009b; Casteel et al. 2009), but in-depth studies on the consequence of a contaminated WB on the contamination level of lettuce processed in consecutive portions has not been documented before. Therefore, the viral transmission from a contaminated WB to three consecutive portions of lettuce was investigated. MS2 bacteriophages and murine norovirus (MNV-1), previously identified as adequate models for human pathogenic enteric viral adhesion on inert and lettuce surfaces (Deboosere et al. 2012), were used as surrogates for human noroviruses (NoV).

Currently, extensive data regarding the practical validation of the importance of cross-contamination during the washing stage, in particular without sanitizers used as is still often the case in some European countries, is missing and has been identified as a data gap in Chapter 6 for accurate risk assessment in the fresh produce supply chain (Danyluk and Schaffner 2011; Zhang et al. 2009). As such, the goal of this cross-contamination study was to produce useful quantitative data on transfers from water to lettuce and to obtain insights on the impact of water management practices in fresh-cut processing on bacterial and viral pathogens' distribution in the fresh produce supply chain.

5.3. MATERIALS AND METHODS

5.3.1. Bacterial strains and inoculum preparation

A three-strain cocktail of *E. coli* previously isolated from lettuce, soil, and fresh-cut processing wash water, was used. The three strains were also deposited to the LFMFP-UGent culture collection: lettuce (nr. 853), soil (nr. 854) and wash water (nr. 855).

A two-strain cocktail of nalidixic acid resistant verotoxin negative (VT-) *E. coli O157* strains (LFMFP nr. 811 and nr. 846) was used. Strain LFMFP 811 was derived from strain CECT 5947 provided by the Group on Quality, Safety and Bioactivity of Plant Foods of CEBAS-CSIC (Spain), whereas strain LFMFP 846 was derived from strain EH1434 from UZ Brussels provided by the Technology and Food Science Department of ILVO (Belgium). Nalidixic acid-resistant (Nal^R) *E. coli O157* cultures were obtained by

consecutive 24 h transfers of BHI cultures to BHI with increasing concentrations of nalidixic acid (Nal) (Merck, Darmstadt, Germany) until isolates were resistant to 50 mg of nalidixic acid per ml.

To obtain appropriate inocula for *E. coli* and Nal^R *E. coli O157*, the separate strains for both inocula were consecutively subcultured twice in respectively 10 ml of nutrient broth and 10 ml of TSB supplemented with nalidixic acid (Nal, 50 mg/ml), and incubated at 37°C until the culture reached the stationary phase. After the second incubation, cultures were mixed, equal volumes of cell suspensions were combined to give approximately equal populations of each culture. The cocktail was centrifuged at 1800 *g* for 10 min at 20°C, washed two times in phosphate buffer, centrifuged again and resuspended in phosphate buffer to obtain a final concentration of approximately 9 log CFU/ml and 8 log CFU/ml for *E. coli* and *E. coli* O157, respectively.

5.3.2. Viral strains and inoculum preparation

A stock of bacteriophage MS2 was obtained in accordance with the standard method of the International Organization for Standardization, (ISO 10705-1), using *Salmonella* Typhimurium WG49 as a bacterial host (Anonymous 1995). Aliquotes of the phage stock suspension were stored at -80°C.

Cells of the murine macrophage cell line RAW 264.7 (ATCC TIB-71; provided by H.W. Virgin, Washington University School of Medicine, St. Louis, MO) were grown in complete Dulbecco's modified Eagle's medium (DMEM) at 37°C under a 5% CO_2 atmosphere. Complete DMEM consisted of DMEM (Lonza, Walkersville, MD) containing 10% low-endotoxin fetal bovine serum (HyClone, Logan, UT), 100 U/mL penicillin, 100 µg/mL streptomycin (Lonza), 10 mM HEPES (Lonza), and 2 mM L-glutamine (Lonza).

RAW 264.7 cells were infected with MNV-1.CW1, passage 7, at a multiplicity of infection (MOI) of 0.05 (MNV-1 to cells) for 2 days. After two freeze-thaw cycles, low-speed centrifugation was used to remove cellular debris from the virus lysate (Wobus et al. 2004). The lysate was stored in aliquots at -80°C. The titer of MNV-1 (PFU/mL) was determined by plaque assay (Wobus et al. 2004).

5.3.3. Fresh-cut lettuce and standardized fresh-cut processing wash water

In all experiments, fresh, unbagged, unprocessed lettuce (*Lactuca sativa* L. var. *capitata*) was used. The lettuce was purchased from a local market in Belgium and transported at <4 °C to the lab for further handling. The outer leaves were removed while the inner leaves were cut into pieces of 3 cm.

Standardized water was used to simulate used fresh-cut produce processing water for WB1. This was obtained by homogenizing 67 g of lettuce along with 200 ml of tap water for 120 s in a stomacher bag containing a filter of approximately 500 µm (VWR). The water was stored at 4°C for 16 h before further handling. On the day of the experiment the standardized water was further diluted with tap water to obtain a chemical oxygen demand (COD) value of approximately 800 mg/L (López-Gálvez et al. 2013). Chemical Oxygen demand (COD) was measured according to the small-scale sealed-tube method (Anonymous, 2002) (LCI 400; Hach Lange; Belgium). When potable municipal water was used this was also pre-cooled at 4°C.

5.3.4. Determination of cross-contamination from lettuce to water using **E. coli** *as an indicator organism*

In order to determine to which extent fecal contaminated lettuce could be the cause for the introduction and maintenance of elevated levels of *E. coli* in the washing baths, fresh-cut lettuce was inoculated with *E. coli*. Lettuce leaves were cut into slices of 3 cm and grouped in portions of 200 g. An inoculum level of ca. 10^4 CFU/g of *E. coli* was obtained by submersion of fresh-cut lettuce for 1 minute (with manual stirring) in 1.5 L of *E. coli* contaminated standardized water (10^5 *E. coli*/ml), drying by a handheld salad spinner, and grouping the fresh-cut lettuce in portions of 200 g and keeping them overnight at 5 $\pm$ 1 °C until further use for the washing experiment.

The simulation of an industrial fresh-cut washing process consisted of two subsequent washing baths filled with initial (at start of the experiment) potable water. First, *E. coli* contaminated lettuce portions of 200 g were washed in a first washing bath (WB1) by manual stirring of 200 g in 4 L of potable water (5 $\pm$ 2 °C, pH = 7.2 $\pm$ 0.2). After one minute the 200 g lettuce was removed from the water, 20 g was retained, spin-dried in a handheld salad spinner for 30 s. The remaining 180 g of lettuce was transferred to a second washing bath (WB2) containing 3.6 L of (initial) potable water (and thus establishing in WB2 as in WB1 a product/water ratio of 0.05 kg/L). The fresh-cut lettuce was again washed by manual stirring for 1 minute. After washing in WB2, the 180 g of lettuce was removed from the water, spin-dried in a handheld salad spinner for 30 s and 20 g was retained for microbial analyses. This procedure was performed for using ten consecutive portions of inoculated lettuce (of 200 g), all ten portions passed subsequently through the same two washing baths. For each portion of lettuce subjected to the simulation of the industrial fresh-cut washing process, 20 gram samples were taken after each washing step and analyzed in double (2 x 10 g) for the presence of *E. coli*. After washing of each 200 g portion of lettuce, 5 ml of wash water was taken to determine the *E. coli* levels in the residual washing water of WB1 and WB2. Also after each portion of 200 g fresh-cut

lettuce being washed, the washing baths were intermediate supplemented with 50 to 90 ml potable water to the initial volume (respectively 4 L for WB 1 and 3.6 L for WB 2) to account for the water losses during the process of fresh-cut lettuce washing and the amount of water taken for testing (approximately 45 - 85 ml being the amount of water being adhered to the portion of fresh-cut lettuce being washed and taking out of the water bath when recovering the lettuce from the washing bath). The whole experiment was conducted in duplicate.

5.3.5. Determination of cross-contamination from water to lettuce using **E. coli** *as an indicator organism*

In this experiment, WB1 containing 4 L of standardized water (5 $\pm$ 2 °C, pH = 7.6 $\pm$ 0.2) was spiked with *E. coli* to evaluate the potential transfer of *E. coli* from the inoculated standardized washing water (without addition of sanitizers) to the fresh-cut lettuce when subjected to a washing procedure as is the case in an industrial fresh-cut washing process. Furthermore, the effect of washing the fresh-cut lettuce subsequently in a second washing bath (WB2), containing initial potable water (3.6 L, 5 $\pm$ 2 °C, pH = 7.2 $\pm$ 0.2) (product/water ratio starting at 0.05 kg/L) on the residual *E. coli* contamination level on the lettuce was established. The *E. coli* cross-contamination from the water to the lettuce was monitored for ten consecutive portions of 200 g of fresh-cut lettuce being subjected to the simulation of the industrial washing process. The *E. coli* contamination of the standardized washing water in WB1 was set in three separate experiments (A, B and C) at approx. 3.0, 4.0 and 5.0 log CFU *E. coli*/100 ml. These levels are indicative for various potential levels of *E. coli* load in the water of the washing baths that may be obtained in a fresh-cut lettuce produce processing company when no sanitizers are used (Holvoet, Jacxsens, Sampers, & Uyttendaele, 2012). For each *E. coli* contamination level of WB1, the experiment was conducted in duplicate. The same experimental approach for washing the fresh-cut lettuce, sampling and analysis was used as mentioned above in section 5.3.4. A schematic representation is depicted in Figure 5.1. Briefly, 5 ml water samples were taken to determine the *E. coli* levels of WB1 and WB2 after each portion of lettuce washed. Lettuce samples (2 x 10 g) were put aside after each washing step for each portion of lettuce involved. After washing one batch, the washing baths were intermediate supplemented with the same water quality initially present in the respective wash baths.

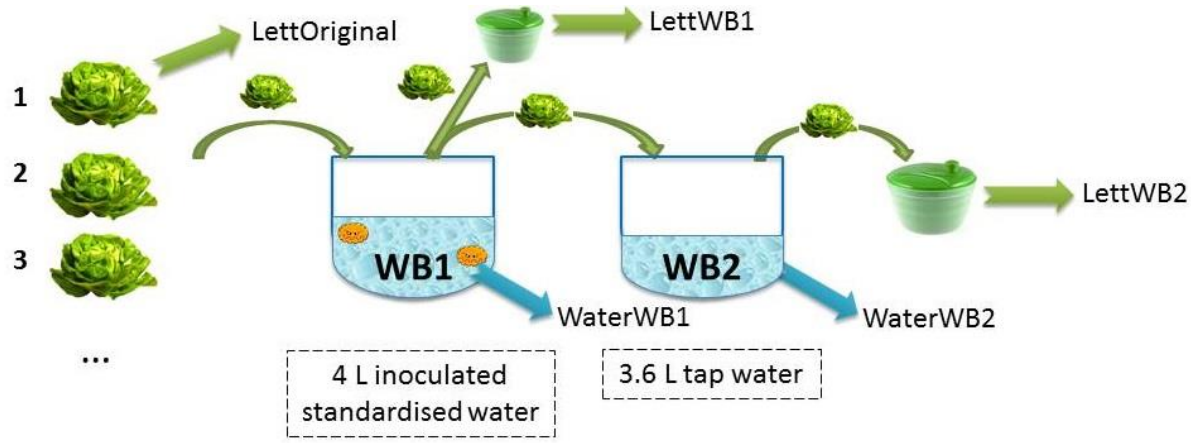


Figure 5.1. Schematic representation of the washing and sampling protocol applied for the determination of bacterial and viral transfer from prolonged used water to fresh-cut lettuce. WB1: washing bath 1; WB2: washing bath 2; Lett: lettuce.

5.3.6. Determination of the effect of a final rinsing step with potable water on residual E. coli contamination of fresh-cut lettuce

This experiment was performed to establish whether rinsing with potable water would be more effective to reduce the introduced *E. coli* load on the washed fresh-cut lettuce (via transfer from the water in WB1) than washing (and thus submersion of the fresh-cut lettuce portion) in a second washing bath with initial potable water. Four portions of 200 g of cut lettuce were subsequently washed in a washing bath with 4 L of inoculated standardized washing water. The experiment was performed with three different inoculum levels of *E. coli* (4.1, 5.2 and 6,4 log CFU *E. coli*/100 ml). However, in this experiment after the first washing bath, the 200 g washed fresh-cut lettuce was not transferred to a second washing bath with (initial) potable water. Instead, the 200 g washed fresh-cut lettuce was subsequently rinsed with a full cone nozzle with potable water for 10 to 20 seconds applying various volumes of rinsing water: the 200 g portions of prior washed (in WB1) fresh-cut lettuce were rinsed with respectively 400, 300, 200 and 100 ml of potable water. Four 10 g samples of the fresh-cut lettuce portion before rinsing and after rinsing were dried by a handheld salad spinner for 30 s and analyzed for *E. coli* numbers. *E. coli* was also enumerated in the water of the WB after every portion of fresh-cut lettuce being washed.

5.3.7. Determination of transfer and cross-contamination of E. coli *O157, MS2 phage and MNV-1 from water to fresh-cut lettuce*

The approach as mentioned in section 5.3.5 for determination and transfer of *E. coli* from water to lettuce was repeated, but with only three portions of fresh-cut lettuce being washed, for the evaluation of the transfer and cross-contamination of *E. coli* O157 (Nal^R, VT-) and MS2 phages. As such, in three independent experiments (experiment A, B and C), the standardized washing water of WB1 (4 L, 5 ± 2°C) was spiked respectively with 4.8, 5.6 and 6.7 log CFU *E. coli* O157/100 ml and 4.0, 5.1 and 6.5 log PFU MS2/100 ml. The WB2 contained initially 3.2 L (product/water ratio starting at 0.05 kg/L) non-inoculated potable water (5 ± 2°C). A single experiment involved the two phase washing of three subsequent portions of lettuce (200 g each) with the washing water of WB1 (10 ml sampled) and WB2 (34 ml sampled) and the fresh-cut lettuce being sampled after passing WB1 and WB2 as described in section 5.3.4. (or Figure 5.1.) and analyzed for the presence of respectively *E. coli* O157 and MS2 phages. This simulation was also repeated with MNV-1 as NoV surrogate virus in a single spiked concentration of 6.5 log PFU/ 100 ml in the standardized washing water in WB1. The pH of the two WBs were measured before the washing of each of the three portions of lettuce and was respectively 7.51 ± 0.14 and 7.88 ± 0.14 for WB1 and WB2.

5.3.8. Bacterial analysis

For *E. coli* and Nal^R *E. coli* O157 enumeration in lettuce, 10 g of lettuce was weighed in a stomacher bag and homogenized for 1 minute in 90 ml peptone water (Oxoid, UK). Tenfold dilution series were prepared in peptone physiological salt and enumerated using the pour plate method on RAPID' *E. coli 2/*Agar (BioRad, France) and on Chromocult coliform-Nal+ agar (Merck) (Nal, 50 mg/ml) for respectively *E. coli* (AFNOR, 2004) and *E. coli* O157. Plates were incubated at 37°C for 18-24 h. Due to the expected low concentration, the enumeration of *E. coli* in water of the washing baths was analyzed according to ISO 9308-1 (i.e. membrane filtration) although the less selective terigtol 7 media was replaced by Rapid' E. coli 2 chromogenic media (Biorad, France) (Anonymous 2000). The enumeration of Nal^R *E. coli* O157 in water was performed by means of tenfold dilution series in peptone physiological salt and the pour plate method with Chromocult coliform-Nal+ agar (Nal, 50 mg/ml) when high contamination levels were expected, and by membrane filtration method when low contamination levels were likely. Both media were incubated at 37°C for 18-24 h.

5.3.9. Viral analysis

The titer of the water of both WBs was directly determined by a double-layer plaque assay (Wobus et al., 2004) without a virus concentration step after storage at -80°C. For virus detection on lettuce samples (10 g), the viral elution-concentration method was used as described before in Chapter 4, although slightly modified (no pectinex in elution buffer and no chloroform/butanol purification step was included). The final virus concentrate (2 ml) was stored at -80°C for the detection of viruses. MS2 phages were detected according to ISO 10705-1 (Anonymous 1995) and MNV-1 was detected by plaque assay as described by Wobus et al. (2004).

5.3.10. Data analysis

SPSS statistics 20 and Microsoft Excel were used for statistical analysis. The Kolmogorov-Smirnov test and Levene's test were used to assess normality and equality of variance respectively. If normally distributed, the difference of the microbial load on the lettuce before and after WB1 was determined by a one sample t-test. A paired T-test was used to check the influence of WB2 or rinsing on the microbial quality of the lettuce. If normality or equality of variance could not be assumed, the Kruskal-Wallis (KW) test was used. Correlations between the contamination of the wash bath and the contamination of the lettuce were checked by means of a spearman rank correlation. p-Values < 0.05 were deemed statistically significant.

The transfer ratio was calculated to quantify the cross-contamination of microorganisms from the inoculated WB1 to the lettuce. A transfer ratio of 100% means that the microbial contamination in WB1 (4 L) is fully transferred to the lettuce (200 g). Meaning that 1 g of lettuce would contain after washing in WB1 an equal amount of microorganisms as initially present in 20 ml of WB1. In reality, only a fraction of the microorganisms present in WB1 is transferred. This transfer ratio was calculated by dividing the mean contamination level of the lettuce (CFU/g) after WB1 by the inoculation level (CFU/100 ml) divided by 5 and by eventually multiplying by 100. For example: if 5.0 log *E. coli*/100 ml was available in the first washing bath, and 1.9 log *E. coli*/g was found on the produce, the product/ water transfer ratio can calculated by means of following formula:

$$100\ \% = \frac{Total\ load\ WB1\ \left(\frac{CFU}{ml}\right) * Volume\ WB1\ (ml)}{weight\ batch\ (g)} = \frac{10^3\ \frac{CFU}{ml}\ * 4000\ ml}{200\ g}$$

$$\rightarrow X\% = \frac{load\ lettuce(\frac{CFU}{g})}{100\ \%\ (\frac{CFU}{g})} = \frac{10^{1.9}(\frac{CFU}{g})}{20000\ (\frac{CFU}{g})} = 0.4\ \%$$

5.4. RESULTS

5.4.1. Determination of cross-contamination from lettuce to water using E. coli *as an indicator organism*

After washing a first 200 g portion of (artificially) contaminated lettuce (ca. 4.0 log *E. coli*/g) in two subsequent WBs (both initially filled with potable water without sanitizers), a rapid transfer and high *E. coli* levels in the washing water of both washing baths were observed: respectively ca. 4.0 and 3.5 log CFU *E. coli*/100 ml for WB1 and WB2 (Figure 5.2.). After washing ten subsequent 200 g portions contaminated fresh-cut lettuce, the *E. coli* load in the washing waters increased up to 5.4 log CFU *E. coli*/100 ml for WB1 and 4.9 log CFU *E. coli*/100 ml for WB2. For the (artificially) contaminated fresh-cut lettuce a mean significant reduction of 0.33 $\pm$ 0.1 ($P < 0.05$, one sample t-test) log CFU *E. coli*/g was observed after passing the WB1. An additional reduction of 0.16 $\pm$ 0.1 ($P < 0.05$, paired t-test) was observed after washing in WB2.

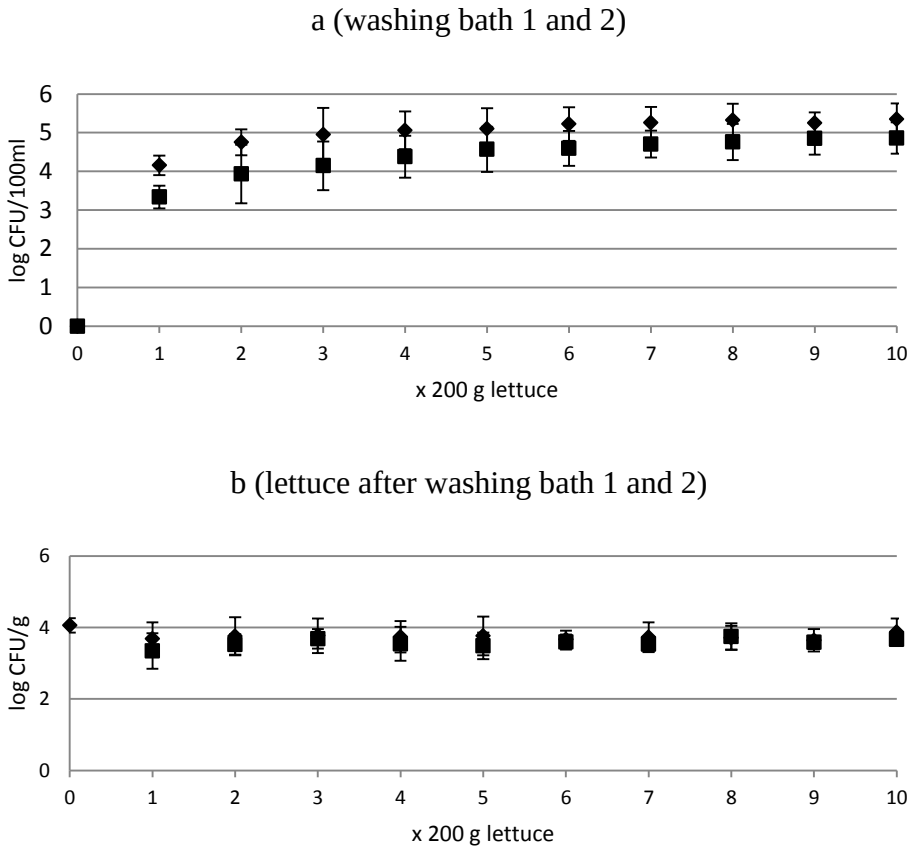


Figure 5.2. *E. coli* contamination of the water (log CFU/100 ml) of WB 1 (♦) and WB 2 (■) (a) and *E. coli* contamination of the (initial 4 log *E. coli* contaminated) fresh-cut lettuce portions (log CFU/g) after passing in WB 1 (♦) and WB 2 (■) (b) in function of the number of 200 g fresh-cut lettuce portions washed in the washing bath (WB).

5.4.2. Determination of cross-contamination from water to lettuce using **E. coli** *as an indicator organism*

Washing fresh-cut lettuce (natural *E. coli* contamination <1 log CFU *E. coli*/g) in standardized washing water simulating prolonged used water without sanitizers in a fresh-cut processing plant (Holvoet et al. 2012) resulted in cross-contamination from the washing water to the washed lettuce. In addition, a rapid increase in *E. coli* contamination of the (initial) potable water in the second washing bath (WB2) was established due to co-transfer of 50 to 90 ml adhered washing water from the WB1 to the lettuce to the WB2. Even at the immediate start of the washing experiment, when the first 200 g portion of washed lettuce was transferred from WB1 to WB2, *E. coli* levels of respectively ca. 1.5, 2.5 and 3.5 log CFU/100 ml were measured in the water of WB2 for the three separate inoculation experiments A, B and C (Figure 5.3.). During the continuation of the experiment, when subsequent (up to 10) 200 g lettuce portions were washed, the *E. coli* levels of the water of WB2 further increased with increasing amounts of lettuce having passed through the WB2.

It can be noted from comparing the three inoculation experiments A, B and C that increasing of the initial *E. coli* inoculum level of the standardized washing water in WB1 is accompanied with a significant increase in *E. coli* contamination on the lettuce ($P < 0.01$, KW) sampled after passing WB1. The contamination on the lettuce ranged from below detection limit (<1.0 log CFU/g) for experiment A (starting inoculum ca. 3.0 log CFU/100 ml) up to 1.9 $\pm$ 0.1 log CFU/g for experiment C (starting inoculum ca. 4.9 log CFU *E. coli*/100 ml) (Figure 5.3.). In case of experiment C, with established enumerable contamination of *E. coli* on the fresh-cut lettuce, a mean significant decrease ($P < 0.05$, paired t-test) of 0.4 log CFU *E. coli*/g was observed on the fresh-cut lettuce portions after passing and submersion for 1 min in the washing water of WB2.

Figure 5.4. shows the correlation between the *E. coli* contamination in the washing bath and the *E. coli* contamination on the washed batch of lettuce. When the wash water of the washing bath is contaminated with 3.6 $\pm$ 0.1 log *E. coli*/100 ml the contamination on the lettuce is near to the detection limit for *E. coli* enumeration (1.0 $\pm$ 0.3 CFU/g). An increase in *E. coli* contamination in the wash bath was accompanied with a linear increase in contamination on the lettuce reaching 3.3 $\pm$ 0.1 log *E. coli*/g for a water contamination of 6.4 $\pm$ 0.1 log CFU *E. coli*/100 ml (Figure 5.4.). This confirmed the data from the previous experiment.

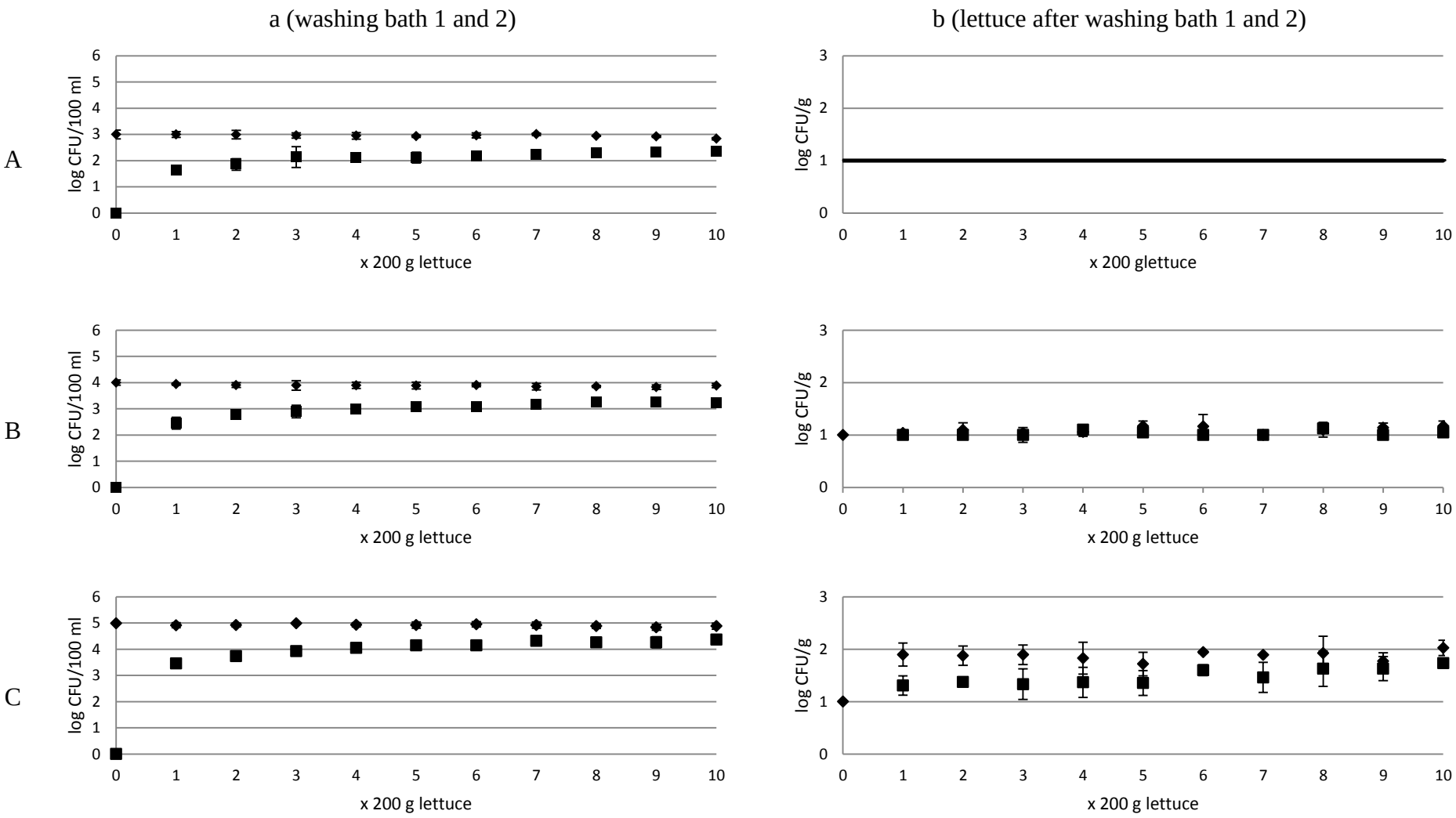


Figure 5.3. *E. coli* contamination of the water (log CFU/100 ml) (a) and *E. coli* contamination of the (initial non-*E. coli* contaminated) fresh-cut lettuce portions (log CFU/g) (b) after passing in washing bath 1 (♦) and washing bath 2 (■) in function of the number of 200 g fresh-cut lettuce portions washed in both washing baths with *E. coli* levels in WB 1 of (A) 3 log CFU *E. coli*/100 ml, (B) 4 log CFU *E. coli*/100 ml, and (C) 5 log CFU *E. coli*/100 ml.

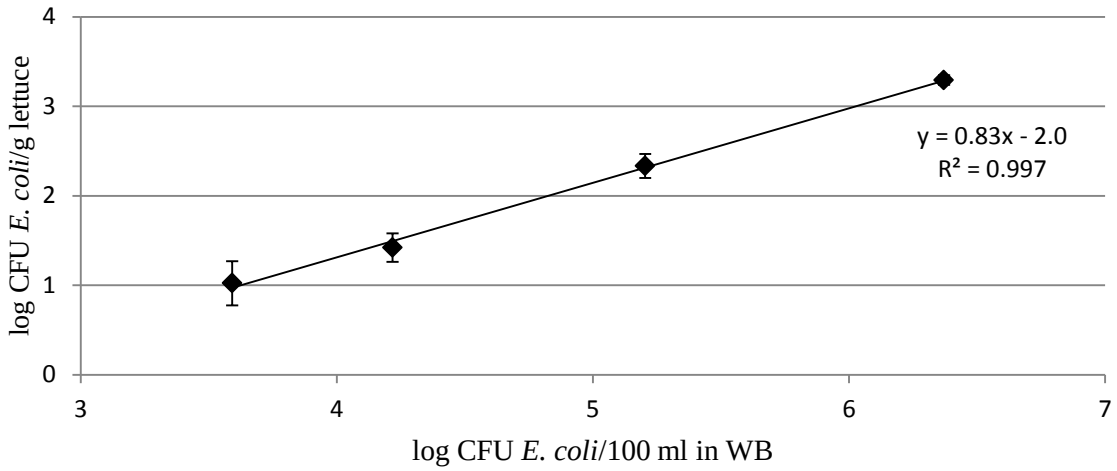


Figure 5.4. Impact of the initial *E. coli* contamination of the water (log CFU *E. coli*/100 ml) in the washing baths (WB1 and WB2) on the resulting *E. coli* contamination level of the lettuce (log CFU *E. coli*/g) after passing the washing baths.

5.4.3. Determination of the effect of a final rinsing step with potable water on residual E. coli *contamination of fresh-cut lettuce*

The reduction of the *E. coli* load on the lettuce by rinsing after washing in the contaminated WB increased from 0.08 $\pm$ 0.07 for a product/water ratio of 2.0 up to 0.21 $\pm$ 0.10 for a product/water ratio of 0.5 (Figure 5.5.). However, despite that the reduction was significant for all produce/water ratios (Paired t-test, $P < 0.05$), the microbial relevance of a 0.2 log CFU/g reduction is negligible (FDA 2001).

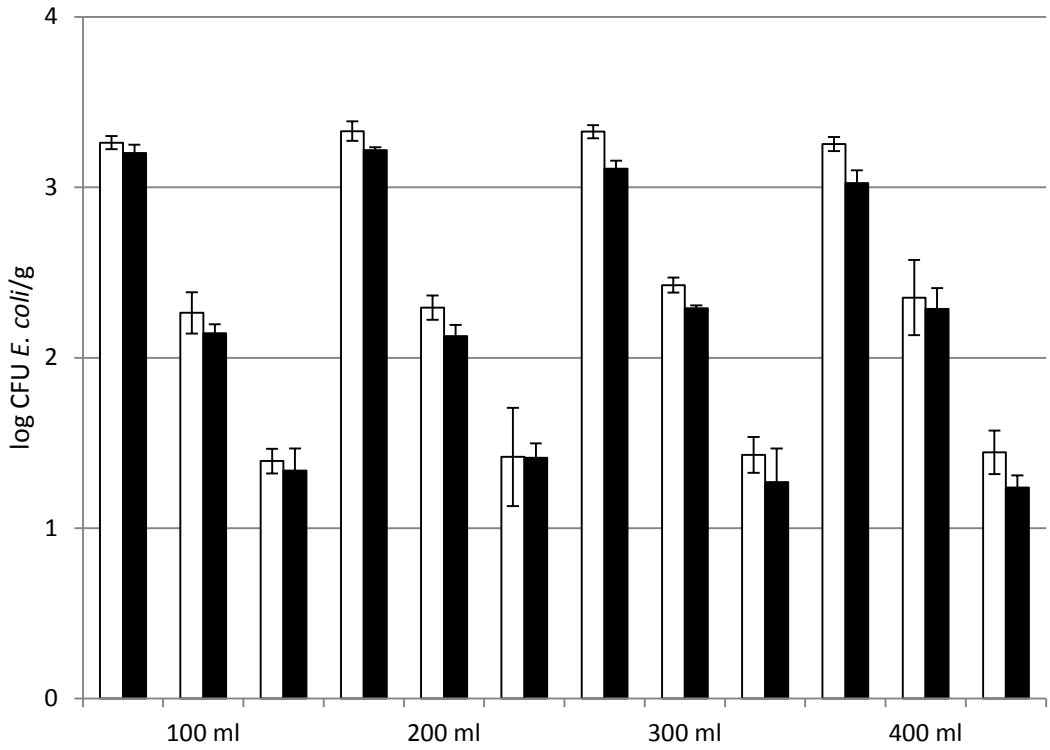


Figure 5.5. *E. coli* levels (log CFU/g) on fresh-cut lettuce portions before (□) and after (■) rinsing (rinsing occurred with 400, 300, 200 and 100 ml of water per 200 g fresh-cut lettuce portion). The fresh-cut lettuce portions were initially contaminated with *E. coli* by cross-contamination via prior washing (submersion with 1 min manual stirring) in a 4 L water bath with contamination levels of respectively 4.1, 5.2, and 6.4 log CFU *E. coli*/100 ml.

5.4.4. Determination of transfer and cross-contamination of E. coli *O157, MS2 phage and MNV-1 from water to fresh-cut lettuce*

For the transfer experiment with *E. coli* O157, WB1 was spiked with an inoculum of respectively 4.8, 5.6 and 6.7 log CFU *E. coli* O157 (Nal^R)/100 ml for three subsequent inoculation experiments A, B and C. The cross-contamination of the lettuce was significant related to the contamination of WB1 (Spearman rank Correlation coefficient 0.94, $P < 0.01$). As such, the higher the inoculation level of WB1, the higher the cross-contamination of the lettuce after passing WB1. This was also visible in Table 5.1. by means of the transfer ratio, as this transfer ratio maintained in the same order of magnitude for the different inoculation levels of WB1. The overall mean transfer ratio of *E. coli* O157 from WB1 to the lettuce was 1.0% ± 0.3% over all inoculation experiments, resulting in a mean transfer of respectively 2.0 ± 0.4, 3.0 ± 0.1 and 3.9 ± 0.1 log CFU/ g lettuce for inoculation experiment A, B and C. After WB2, there is a significant decrease in contamination level of the lettuce ($P < 0.01$, paired t-test) compared to the contamination level of lettuce only washed in WB1. The mean reduction of contamination level of *E. coli* O157 on the lettuce after WB2 was 0.9 $\pm$ 0.3 log CFU *E. coli* O157/g of lettuce over all

inoculation experiments for *E. coli* O157. During each inoculation experiment, the contamination level of WB2 gradually increased during the washing of the three lettuce portions until a level approx. 0.9 ± 0.1 log lower compared to the contamination level in WB1.

For the transfer experiment of MS2 phages from the wash water to the lettuce, the inoculum level of MS2 in WB1 was respectively 4.0, 5.1 and 6.5 log PFU/ 100 ml for experiment A, B and C. The overall mean transfer ratio of MS2 from WB1 to the lettuce was 0.5% ± 0.2% over all inoculation experiments, resulting in contamination of respectively 0.7 ± 0.4 log PFU/g, 2.1 ± 0.2 log PFU/g and 3.7 ± 0.1 log PFU/g lettuce for inoculation experiment A, B and C. Thus, the extent of the MS2 cross-contamination from WB1 to the lettuce was – similar to *E. coli* O157 – related to the contamination level of WB1 as an increase in contamination level of WB1 resulted in a higher cross-contamination level to the lettuce. Further processing of the lettuce in WB2 significantly reduced ($P < 0.01$, paired t-test) the MS2 contamination level of the lettuce in each portion with approx. 0.9 ± 0.3 log PFU in inoculation experiment B and C. Results from inoculation experiment A were not included as the microbial load of the lettuce after WB2 was below the detection limit in two out of three portions. However, for inoculation experiment C, it is discernible that the effect of WB2 in reducing the microbial level of the processed lettuce portions, showed a slight downward trend as more consecutive portions of lettuce were processed and hence the contamination level of WB2 rose till its final level of approx. 0.7 log PFU/100 ml lower than the contamination level in WB1 (Table 5.1.).

For MNV-1, the inoculation experiment involving a contamination level of WB1 of 6.5 log PFU/100 ml showed that the contamination level of WB2 approached the contamination level of WB1 up to a level of approx. 1.4 log PFU/100 ml. The mean transfer ratio of MNV-1 from WB1 to the lettuce was 0.5% ± 0.1% resulting in a mean contamination level of 3.4 ± 0.1 log PFU/g lettuce after WB1. Noteworthy, a significant reduction of approx. 1.3 ± 0.2 log PFU/g lettuce was observed after washing the lettuce in WB2 ($P < 0.01$, paired t-test).

Table 5.1. *E. coli, E. coli* O157, MS2, and MNV-1 on lettuce leaves and in processing water after washing of each lettuce portion for the different inoculation levels of WB1.

Micro-organism	Inoculums level	N° of lettuce portion	Log CFU or PFU/100ml in WB1	Log CFU or PFU/g on lettuce after WB1 (X%)	Log CFU or PFU/100ml in WB2 (Δ^a)	Log CFU or PFU/g on lettuce after WB2 (Δ^b)
E. coli	A	0	2.9	<1	<0	<1
		1	2.9	<1	1.6 (1.3)	<1
		2	2.9	<1	1.9 (1.0)	<1
		3	2.9	<1	2.1 (0.8)	<1
		10	2.9	<1	2.3 (0.6)	<1
	B	0	4.0	<1	<0	<1
		1	3.9	1.0±0.2 (0.7%)	2.4 (1.5)	<1
		2	3.9	1.1±0.2 (0.8%)	2.8 (1.1)	<1
		3	3.9	1.0±0.2 (0.7%)	2.9 (1.0)	<1
		10	3.9	1.2±0.2 (0.9%)	3.2 (0.7)	1.1±0.1 (0.1±0.1)
	C	0	5.0	<1	<0	<1
		1	4.9	1.9±0.2 (0.5%)	3.5 (1.4)	1.3±0.2 (0.6±0.2)
		2	4.9	1.9±0.2 (0.5%)	3.7 (1.2)	1.4±0.1 (0.5±0.2)
		3	5.0	1.9±0.2 (0.5%)	3.9 (1.1)	1.3±0.3 (0.6±0.1)
		10	4.9	2.0±0.1 (0.6%)	4.4 (0.5)	1.7±0.1 (0.3±0.1)
***E. coli* O157**	A	0	4.9	ND	<0	ND
		1	4.9	2.2* (1.1%)	3.4 (1.5)	1.0±0.0 (0.6±0.8)
		2	4.8	1.9±0.0 (0.6%)	3.5 (1.3)	1.0* (0.9±0.0)
		3	4.7	2.0±0.0 (0.8%)	3.8 (0.9)	<1
	B	0	5.5	ND	<0	ND
		1	5.6	3.1±0.1 (1.5%)	4.2 (1.4)	1.8±0.2 (1.3±0.2)
		2	5.7	3.0±0.1 (1.1%)	4.5 (1.2)	2.0±0.1 (1.0±0.1)
		3	5.6	2.9±0.0 (1.0%)	4.6 (1.0)	2.1±0.2 (0.8±0.2)
	C	0	6.6	ND	<0	ND
		1	6.7	4.0±0.1 (0.9%)	5.2 (1.5)	2.9±0.0 (1.1±0.1)
		2	6.8	3.9±0.1 (0.8%)	5.5 (1.3)	3.0±0.1 (0.9±0.1)
		3	6.7	3.9±0.1 (0.8%)	5.9 (0.8)	3.2±0.1 (0.7±0.1)
MS2 phage	A	0	4.1	ND	<2	ND
		1	3.9	1.8* (0.3%)	2.0 (1.9)	<0.3
		2	3.9	0.7±0.5 (0.3%)	2.8 (1.1)	<0.3
		3	4.0	0.7±0.5 (0.3%)	3.0 (1.0)	0.3±0.0 (0.4±0.5)
	B	0	5.1	ND	<2	ND
		1	5.1	2.0±0.1 (0.4%)	3.3 (1.8)	1.1±0.1 (0.9±0.1)
		2	5.1	2.0±0.0 (0.4%)	3.7 (1.4)	1.1±0.5 (0.9±0.5)
		3	5.2	2.3±0.0 (0.7%)	3.9 (1.3)	1.3±0.4 (1.0±0.4)
	C	0	6.6	ND	<2	ND
		1	6.6	3.8±0.0 (0.9%)	5.2 (1.4)	2.4±0.1 (1.4±0.1)
		2	6.5	3.6±0.0 (0.6%)	5.4 (1.0)	2.8±0.2 (0.8±0.2)
		3	6.4	3.7±0.1 (0.8%)	5.7 (0.7)	3.1±0.0 (0.6±0.1)
MNV-1	A	0	6.4	ND	<2.3	ND
		1	6.4	3.5±0.0 (0.5%)	4.7 (1.7)	2.0±0.0 (1.5±0.0)
		2	6.5	3.4±0.0 (0.5%)	4.8 (1.7)	2.1±0.1 (1.3±0.1)
		3	6.5	3.3±0.0 (0.4%)	5.1 (1.4)	2.0±0.2 (1.3±0.2)

'a': difference (Δ) in log units/100 ml between the contamination level of WB1 and WB2

'b': the reduction (Δ) in log units/g lettuce of the contamination level of the lettuce after washing in WB2 compared to lettuce only washed in WB1.

Detection limit of *E. coli, E. coli* O157, MS2, and MNV-1 in water is respectively 0 log CFU/100 ml, 0 log CFU/100ml, 2.0 log PFU/100 ml, and 2.3 log PFU/100 ml water; in lettuce the detection limit is respectively 1 log CFU/g lettuce, 1 log CFU/g lettuce, 0.3 log PFU/g lettuce and 0.3 log PFU/g lettuce.

ND: not determined

'*': one of both duplicates had a concentration below detection limit in lettuce. In this case only the concentration of the positive sample was mentioned and used for calculations.

5.5. DISCUSSION

In the study, a fresh-cut lettuce washing process was simulated through a dynamic process using two washing steps. The microbial load of both wash water and lettuce were measured after each portion was washed. This study distinguishes itself from previous studies by the measurement of the microbial load of both water and produce, the absence of sanitizers in the washing baths, and the implication of both bacteria and viruses in a dynamic two-step wash process (Allende et al. 2008; Baert et al. 2008c; Croci et al. 2002; Lopez-Galvez et al. 2010b; Luo 2007; Luo et al. 2011).

In general, washing systems for any fresh-cut vegetable processing consist of two or three separate washing stages (FSAI 2001). The first WB aims to eliminate general field dirt and debris. Consequently, the organic and microbial load of this washing water may increase rapidly. The purpose of the subsequent WB includes the reduction of the microbial load from the lettuce leaves. The aim of this research was to provide more quantitative information about the bacteriological and viral transfer from water in these WBs to lettuce and *vice versa* during a washing process in order to gain insight into a proper water management in the case study when sanitizers are not used either voluntarily or prohibited under national legislation (Artes et al. 2009; Rico et al. 2007). Furthermore, these data may gain insights useful for future risk assessment and management (Danyluk and Schaffner 2011). In this study the microbial load was represented and monitored by inoculation and enumeration of hygiene indicator *E. coli*, the pathogen *E. coli O157*, and MS2 bacteriophage and MNV-1 as surrogates for human NoV.

The numbers of *E. coli* on unprocessed lettuce may vary from below the detection limit (<10 CFU/g) to approximately 4 log CFU/g (Arthur et al. 2007; Aycicek et al. 2006; Boraychuk et al. 2009; Mukherjee et al. 2006) with *E. coli* levels >100/g indicated overall as marginal quality and of >1000/g as unacceptable quality (EC Regulation 2073/2005, 2005) (Anonymous 2005). Overall, due to a low infectious dose of both pathogenic *E. coli* and norovirus the absence of these pathogens per 25 gram should be warranted. For NoV, most contamination levels that have been found on leafy greens range from 0 to 3 log genomic copies/g leafy greens (Baert et al. 2011; Kokkinos et al. 2012). The microbial load in the washing water of the first washing bath used in fresh-cut processing is predominantly affected by the initial microbial quality of the crops upon arrival from the field at the processing factory (Allende et al. 2008). In the present study it was shown that if highly contaminated (4 log *E. coli* CFU/g lettuce) lettuce crops were submitted to the washing baths, a rapid cross-contamination from the fresh-cut lettuce to the washing water of the WBs occurred. Several studies have investigated viral transfer to washing water originating from strawberries (Casteel et al. 2009) and lettuce (Baert et al. 2009b). In the

latter study, washing of 50 g of viral contaminated iceberg lettuce (inoculums: approx. 6.7 log PFU MNV-1) for 5 min in 500 ml (product/water ratio of 0.1 kg/L) of tap water resulted in a viral contamination level of 3.73 ± 0.06 log PFU/ml of wash water.

Notwithstanding the artificial contamination and thus probably less adherent (or internalized) bacterial cells being present, the mean decrease of the *E. coli* load on the lettuce after washing in the two WBs was only 0.5 log CFU/g which is lower than the 1 to 2 log reductions mentioned in other publications (Lopez-Galvez et al. 2010b; Ragaert et al. 2010). Potable water is able to remove microorganisms to some degree, the process can be slightly enhanced by the use of sanitizers for disinfection (Beuchat 1998; Van Haute et al. 2013a). Water in both WBs can become microbiologically contaminated depending on the quality of the incoming fresh produce from the field.

Moreover, once the washing water is contaminated with *E. coli*, and in the absence of sanitizers, there is opportunity for further spread and cross-contamination of the microbial load from the washing water to the subsequent fresh-cut lettuce portions subjected to the washing process. Contamination levels exceeding 4 and 3 log *E. coli*/100 ml washing water for respectively WB1 and WB2 resulted in elevated levels of *E. coli* on the washed fresh-cut lettuce, even after the processing of only one portion of lettuce (200 g lettuce in 4 L washing water, a product/water ratio of 0.5 kg/L). Concentrations higher than 5 log *E. coli*/100 ml resulted in levels exceeding 2 log *E. coli* CFU/g of lettuce. The results obtained by these experiments are supported by the real life study of Holvoet et al. (2012) who observed *E. coli* levels higher than 2 log CFU/g on processed lettuce when concentrations of 5 log CFU/100 ml were observed in the wash water of a fresh-cut lettuce plant in operation. In the same field study it was also observed that concentrations exceeding 4 and 3 log *E. coli*/100 ml resulted in elevated *E. coli* levels on the processed lettuce. However, lower concentrations in the water does not imply the absence of *E. coli* on the lettuce as no enrichments were conducted in the study. Similar contamination results were obtained for *E. coli* O157. Regardless of the inoculation level of the water in WB1, a rapid increase of the *E. coli/E. coli* O157 loads in WB2 was also observed in the presented study. This latter can be explained by the transfer of drain water attached to the lettuce during transfer from the first to the second WB. After a rapid initial increase, the contamination of WB2 further augmented during the washing process to approximately 1 to 0.5 log below the inoculation level of WB1 after processing of respectively 0.6 kg or 2 kg of lettuce. A similar trend in transfer between both washing baths was observed for the viruses.

Besides the cross-contamination between the WBs, the non-contaminated incoming lettuce was contaminated via the inoculated WB1 as well. The degree of contamination of the

lettuce depended on the inoculation level and increased at higher contamination levels of WB1. This means that, if no sanitizers are allowed, a highly concentrated point contamination with *E. coli* (e.g. bird feces attached to a crop of lettuce) or viruses (from human feces) is able to introduce *E. coli* or other pathogens in the processing water. Through analysis of the transfer ratios it became also clear that only a very small fraction of the micro-organisms (≤1.5%) was transferred from the water phase to the lettuce and this as well for bacteria as for viruses. Even though this transfer ratio is low, point contaminations with *E. coli* or viruses on the fresh produce can easily result in high contamination levels in the subsequent WBs and hence in a high level of cross-contamination to the washed lettuce. For example for *E. coli* this implicates that (assuming that 1 g of bird feces contains ca. 8 log CFU *E. coli*/g (Anderson et al. 1997; Fogarty et al. 2003; Haack et al. 2003; Roll and Fujioka 1997), 1 g of bird feces attached to the crop(s) of lettuce subject to washing in a water tank of approximately 500 liter water is able to contaminate the potable water in the washing bath up to more than 4 log CFU of *E. coli*/100 ml and can thus contaminate the whole lettuce batch. NoVs can be present in human feces in high concentrations up to 6 x 10^{10} genomic copies/g of stool (Richards et al. 2004) and can be transferred to the lettuce at a pre-harvest stage (e.g. due to contaminated irrigation water) or during harvesting because of unhygienic practices (Seymour and Appleton 2001). Due to a combination of high level fecal shedding, high virus stability and low infectious dose, viruses are identified justly as one of the hazards concerning leafy green vegetables (FAO/WHO 2008a).

The use of an additional washing step or rinsing step (with potable water) after the initial washing process did not provide any relevant microbial reduction of established bacterial or viral concentration of the washed fresh-cut lettuce. The ability to attach strongly to the leaf epidermis even after only a one minute dip in a washing tank, was observed for *E. coli* in previous studies (López-Gálvez et al. 2010; Shaw et al. 2008). Remark that even between different strains of a pathogen differences can occur related to attachment characteristics as was demonstrated for the attachment of 'curli producing' and 'non-curli producing' strains of *E. coli* O157 to fresh produce (curli fimbriae is a component of extracellular matrix of *E. coli*, promoting bacterial attachment) (Boyer 2006; Patel et al. 2011). However despite these possible variability in attachment characteristics that hence could possibly influence the transfer rates calculated in this study, the present study does provide fundamental evidence concerning the potential for cross-contamination in washing baths and provides baseline transmission data for further risk assessment that include the potential danger of cross-contamination during the washing process in absence of appropriate sanitizers. Besides the bacterial-surface structures that play a role in the bacterial attachment (Shaw et al. 2008), the virus-specific factors (e.g. virus pI, presence of

food-specific ligands), food (surface) factors (e.g. presence of virus-specific ligands, access to food interior) and extrinsic factors (e.g. pH and presence of substances competing for binding) (Le Guyader and Atmar 2008) play a role in viral attachment/adsorption to (food) surfaces. The interaction of pathogens with produce surfaces can consists of a physical entrapment below the surface in stomata and cut edges (Esseili et al. 2012a; Seo and Frank 1999; Solomon and Matthews 2006; Wei et al. 2010). The latter, could create protective shelters, making washing ineffective (Seo and Frank 1999; Singh et al. 2002). Concerning the virus/food specific binding it was also shown in an experiment performed by Esseili et al. (2012a) that virus-like particles (VLPs) of humane NoV GII.4 can specifically bind to lettuce plant leaves mainly through the carbohydrates of the cell wall next to minor binding to cell wall proteins. Virus adsorption/binding to lettuce has been found to vary depending on the viral strain (Vega et al. 2005) and type of lettuce (Gandhi et al. 2010). As such, the reducing effect of a second phase washing in WB2 on the level of viral and bacterial contamination on the lettuce was irrelevant suggesting that the main priority should be to avoid cross-contamination and distribution of micro-organisms during the washing process knowing that the adherence is likely higher on injured surfaces (cut) than not injured surfaces (Esseili et al. 2012a; Han et al. 2000a; Takeuchi et al. 2000) and even more difficult to remove (Baert et al. 2009a; Han et al. 2000b; Keskinen and Annous 2011).

These results emphasize the vulnerability of a washing process for microbial contamination when no sanitizers are used. The use of merely potable water for washing soiled or contaminated crops as a prerequisite is not adequate from a food safety perspective. Currently, the fresh produce companies in some European countries where sanitizers are prohibited rely mostly on the initial microbial quality of the crops upon arrival from the field and excessive amounts of potable water (Olmez and Kretzschmar 2009). However, these results suggest that a systematic refilling of the water in WB2 provides little benefit to maintain potable water quality if the first WB is contaminated and will act as a vehicle for transmission of microorganisms from contaminated water to lettuce. This is confirmed by other studies showing that the risk of cross-contamination is not removed by using large quantities of water (Holvoet et al. 2012; López-Gálvez et al. 2009). Considering the sustainable use of water, the use of excessive amounts of water for washing of fresh produce should be avoided as availability of potable water is restricted and costly in many areas of the world (Menzel and Matovelle 2010; Parish et al. 2012).

To limit the water use and tackle the risks of cross-contamination, a disinfectant agent even at low concentrations for maintaining acceptable water quality could be considered (López-Gálvez et al. 2010; Van Haute et al. 2013a). The main effect of sanitizing treatments for washing fresh-cut produce is indeed aimed at reducing and controlling the

microbial load of the water used in fresh-cut processing and thus prevent the transfer of micro-organisms (including *E. coli*, enteric pathogens and viruses) from contaminating the fresh-cut end product rather than having a decontamination or preservative effect on the produce itself (Baert et al. 2009b; Gil et al. 2009; Van Haute et al. 2013a; Zhang et al. 2009).

CHAPTER 6

LESSONS LEARNED FROM MICROBIAL RISK ASSESSMENT RELATED TO USE OF WATER AND SAFETY OF FRESH PRODUCE

De Keuckelaere, A., Jacxsens, L., Amoah, P., Medema, G., McClure, P., Jaykus, L-A., Uyttendaele, M. Zero risk does not exist: Lessons learned from microbial risk assessment related to use of water and safety of fresh produce
Submitted to Comprehensive Reviews in Food Science and Food Safety

TABLE OF CONTENTS

Authors contributions

Conception, planning, and interpretation of the paper was done by Liesbeth Jacxsens, Philip Amoah, Gertjan Medema, Peter McClure, Lee-Ann Jaykus, and Mieke Uyttendaele, followed by organizing, collection of relevant literature, interpretation, and writing performed by Ann De Keuckelaere, Liesbeth Jacxsens, and Mieke Uyttendaele and major contributions on rephrasing and writing style made by Lee-Ann Jaykus.

6. LESSONS LEARNED FROM MICROBIAL RISK ASSESSMENT RELATED TO USE OF WATER AND SAFETY OF FRESH PRODUCE

6.1. ABSTRACT

In order to further grasp the current knowledge on water as a transmission route for viral pathogens, a review was performed in this Chapter on the available quantitative microbial risk assessment studies in peer-reviewed literature that included the modeling of effect of water use (e.g. influence of irrigation water and/or washing step) or water treatment on the quality of fresh produce in at least one stage of the farm-to-fork supply chain. This review was not limited to the risk implied by the presence of viral pathogens, since also pathogenic bacteria, parasitic protozoa and helminths were included. This to have a better idea on the modeling strategies generally applied in quantitative microbial risk assessments (QMRA), and hence the current knowledge, for the transfer of pathogens in water to fresh produce.

Risk assessment related to use of water and safety of fresh produce orient from both water and food microbiology studies. Although the set-up and methodology of risk assessment in these two disciplines may differ, analysis of the current literature reveals some common outcomes. Most of these studies from water perspective focus on enteric virus risks, largely because of their anticipated high concentrations in untreated wastewater and their recalcitrance to common wastewater treatments. Risk assessment studies from the food perspective rather focus on bacterial pathogens such as *Salmonella* and pathogenic *E. coli*. Few site-specific data points were available for most of these microbial risk assessments, meaning that many assumptions were necessary which are retaken in many studies. Specific parameters lacking hard data included rates of pathogen transfer from irrigation water to crops, pathogen penetration, and survival in or on food crops. Data on these factors have been investigated over the last decade and this should improve the reliability of future microbial risk estimates. However, the sheer number of different foodstuffs and pathogens, combined with water sources and irrigation practices, means that developing risk models that can span the breadth of fresh produce safety will be a considerable challenge. The new approach using microbial risk assessment is objective and evidence-based and leads to more flexibility and enables more tailored risk management practices and guidelines. Drawbacks are however capacity and knowledge to perform the microbial risk assessment and the need for data and preferably data of the specific region.

6.2. INTRODUCTION

Fecal contaminated irrigation water has been implicated as either a possible source, or a likely source of pathogen contamination of fresh, raw consumed fruits and vegetables (e.g. Ensink et al. 2007; Leifert et al. 2008; Okafo et al. 2003; Thurston-Enriquez et al. 2002). This can lead to foodborne illness and widespread outbreaks. Water used for irrigation may originate from multiple sources and include rain water, ground water, surface water, (treated) wastewater or even desalinated seawater. The availability of water sources for irrigation is under increasing pressure**.** Reconditioned waste or surface water are two abundant sources with potential to replace untreated ground or rain water. Application of alternative water sources may result in an elevated probability of the presence of pathogens and increases the pressure on governing water quality (WHO 2006). Guidelines or even criteria on the quality of water applied for irrigation in fresh produce production are set by some countries or individual states e.g. Canada (Steele and Odumeru 2004), Spain (Iglesias et al. 2004), California (USA) (California Code of Regulations, Title 22, Division 4, Chapter 3)). Most guidelines are empirically derived fixed microbial standards focusing on defined indicator organisms or pathogens. Risk assessment strategies to underpin management of health risks are evidence-based and may also be helpful providing flexibility in setting guidelines for specific situations. In recognition of this, WHO has replaced the original approach of water quality testing for fecal coliforms to evaluate compliance with a guideline of <1000 fecal coliforms per 100 mL (Blumenthal and Peasey 2002) by a risk assessment/risk management based approach with more flexible guidelines. This new approach is based on attributable risks and disability-adjusted life years clarified in the WHO guidelines for use of wastewater in agriculture (WHO 2006). These guidelines provide the framework for national and local decision making to manage the health risk from hazards associated with (treated) wastewater or other alternative sources of water use in agriculture. A similar approach was used in establishing Australian guidelines for water recycling (NRMMC-EPHC-AHMC 2006; O'Toole et al. 2010).

Aside from irrigation water, washing of produce at harvest, during further processing or during preparation may also function as a means of foodborne pathogen contamination of produce (Gil et al. 2009; Chapter 5). In postharvest practice, both the prevention of cross-contamination during the washing process by applying sanitizers, and the reconditioning of spent water for subsequent reuse, have been extensively studied (Van Haute et al. 2013a and 2013b; Lopez-Galvez et al. 2009). However, if occasional contamination does occur, even with adequately operated and monitored washing procedures, microbial concentrations are reduced by only 1 to 2 log units at best (Beuchat 1998). The inclusion of a washing step can therefore result in an increase or decrease on the occurrence of contaminated crops or fresh-cut produce, but its efficacy will depend upon initial pathogen

load as well as the ability to maintain the washing water quality being used during processing and preparation.

The selection of water source, water treatment, and water quality for use throughout the fresh produce supply chain must consider a wide variety of crops, production practices and consumption patterns. Therefore, a flexible approach must be applied in setting microbiological guidelines or criteria for types and uses of water. The principal aim of Microbial Risk Assessment (MRA) is to support risk management by providing an objective, transparent, evidence-based assessment of the health risk of (different) exposure pathways/scenarios. In the case of water use in fresh produce primary production, risk assessment crosses two scientific disciplines, those being environmental (water) science and food science. Although epidemiological studies (observing exposed and non-exposed populations) may also be used to assess risk, and some of these have been carried out to assess risks associated with drinking water, they are costly and the logistics, limited sensitivity in measuring disease, and specific populations being studied mean that quantitative MRAs are often preferred.

The first MRAs for water use in fresh produce production were initiated by risk assessors with a 'water'-background that investigated the contaminated fresh produce as one possible exposure pathway for microbial contaminated reclaimed water (e.g. in Asano et al. 1992; Tanaka et al. 1998). The focus of these initial MRA studies was treatment of waste water and irrigation practices at the farm level. Gradually studies became available that focused on other parts of the fresh produce supply chain, including washing and cutting of fresh produce (e.g. Carrasco et al. 2010; Rodriguez et al. 2011) and integrating the role of consumer preparation (e.g. Domenech et al. 2013). These latter MRAs were executed by risk assessors with a background in food science. As both scientific disciplines have a different perspective and developed their own approach towards MRA, cross-pollination between different disciplines is recommended to expand expertise and promote collaborative understanding (O'Toole et al. 2014). The aim of the present study is to review environmental and food science MRA studies on water and safety of fresh produce to develop a holistic assessment from source water in the farm-to-fork chain where water is included as potential vehicle for foodborne pathogens.

During the review specific consideration is given to production (e.g. irrigation water) or microbial removal strategies (e.g. washing). The selected quantitative MRAs (QMRA) were further analyzed in-depth to: (1) obtain insights in overall approaches used during QMRA modeling; (2) identify investigated mitigation strategies by scenario analysis; (3) summarize assumptions made and surrogate data; (4) identify recurring data gaps; and (5) characterize how risk is expressed and, if applicable, compare with acceptable levels of

protection targets. Finally, lessons learnt and recommendations for future risk assessment studies are defined.

6.3. MATERIALS AND METHODS

6.3.1. Screening of peer reviewed literature and collection of QMRA publications

Relevant publications from peer reviewed literature were selected on the basis of the following criteria: **(i)** a quantitative risk assessment or exposure model calculating the likelihood of infection, illness or presence of **(ii)** a defined microbial foodborne pathogen (bacteria, viruses, protozoa, or helminths) **(iii)** through the consumption of fresh produce or occurrence on fresh produce, and **(iv)** which included the modeling of effect of water use or water treatment on the quality of fresh produce in at least one stage of the farm-to-fork supply chain. Water could have a role in the transmission of the foodborne pathogen to fresh produce during irrigation with contaminated water or during the post-harvest washing process (in fresh-cut processing) or salad preparation. QMRA articles were identified by searching Web of Science™ Core Collection and further search by screening the reference list of identified relevant QMRA articles. Studies that were not selected included one in which a QMRA was done based on use of urine as irrigation water (Hoglund et al. 2002), and one that failed to give specific detail on how the risk calculation was performed (e.g. Aiello et al. 2013). Other QMRAs dealing with the safety of fresh produce but that did not include water in any step of the model (e.g. Franz et al. 2010; Verhaelen et al. 2013a) were also excluded. The collection of publications ended in December 2013.

6.3.2. Classification of selected QMRA publications

In total forty one QMRA studies were selected (Table 6.1.). Studies were classified according to the target pathogen(s) under investigation, which could be either a foodborne virus, parasitic protozoon, bacterium or helminth. A sub-classification was made to describe in which part of the farm-to-fork continuum (production, packing/processing including distribution, consumer home) the impact of water was considered. In case irrigation water was included, it was notified if the study also included the effect of prior water treatment on irrigation water quality. Studies were also sub-classified according to the background/perspective of the risk assessment team. This was done by searching in the affiliations of the authors with the terms ‘water’, ‘environmental’, ‘food’ and ‘agriculture’. When ‘water’ or ‘environmental’ was present for one or more of the name(s), the article was classified as written from a ‘water-perspective’. When ‘food’ or ‘agriculture’ was present, the article was classified as written from a ‘food’ perspective. When the author

names included terms from both groups, the article was classified as written from a 'water and food perspective'. A final sub-classification for the studies was made based on the type of QMRA that was performed: deterministic or stochastic risk assessment.

6.3.3. In-depth analysis of selected QMRA publications

The selected QMRAs were analyzed in-depth to summarize the overall approaches taken in modeling, and the use of assumptions and surrogate data in an effort to identify recurring data gaps. Such data gaps, assumptions, and surrogate data dealt with issues such as: **(1)** pathogen contamination prevalence data; **(2)** transfer rates for pathogens from water to produce; **(3)** behavior (growth, survival, inactivation, removal) of microorganisms in the environment and produce; **(4)** consumer behavior and consumption patterns; and **(5)** dose-response information. The diversity in risk end-point and characterization/benchmarking, such as the use of a tolerable or acceptable risk level, are also identified and discussed.

Results on the outcomes of the QMRA studies are discussed in a second part of this review. This includes the major lessons learned about the intervention strategies or control measures that were investigated relative to water use in the fresh produce supply chain. Also future perspectives in risk assessment related to water and the safety of fresh produce are discussed.

Table 6.1. Classification of the selected QMRA studies according to the target pathogen, the part in the farm-to-fork chain in which the effect of water was included, background of the involved research groups, and type of QMRA.

	Pathogen under study														The step in which the effect of water was modeled in the farm-to-fork chain of fresh produce							
RA studies	Norovirus	Rotavirus	Hepatitis A virus	Enterovirus	Enteric virus	*Giardia* spp.	*Cryptosporidium* spp.	*Entamoeba histolytica*	*Campylobacter*	*L. monocytogenes*	Pathogenic *E. coli* [c]	*Salmonella*	Enteric path. bacteria	Ascaris	Water treatment	Irrigation	$\text{Washing}_{\text{Processing}}$	$\text{Washing}_{\text{consumer}}$	Water-perspective	Food-perspective	Deterministic RA	Stochastic RA
Asano et al. 1992					X										X	X			X		X	
Shuval et al. 1997		X	X													X		X*	X		X	
Tanaka et al. 1998					X										X	X			X			X
van Ginneken and Oron 2000					X										X	X			X			X
Petterson and Ashbolt 2001					X											X			X			X
Petterson et al. 2001a; Petterson et al. 2002				X												X			X			X
Stine et al. 2005b			X									X				X			X	X	X	
Hamilton et al. 2006a					X											X			X			X
Hamilton et al. 2006b					X											X			X			X
NRMMC-EPHC-AHMC 2006; O'Toole et al. 2010		X[a]					X		X							X			X		X	
Mara et al. 2007		X					X		X							X			X			X
Bastos et al. 2008		X				X	X		X							X			X		X	
Diallo et al. 2008						X	X				X					X			X			X
Seidu et al. 2008		X												X		X	X		X			X
Finley et al. 2009													X			X			X		X	

Mota et al. 2009						X	X									X				X	X	
Navarro et al. 2009														X				X	X		X	
Al-Juaidi et al. 2010					X											X			X			X
Barker-Reid et al. 2010					X											X[d]			X			X
Carrasco et al. 2010										X							X			X		X
Forslund et al. 2010		X														X			X	X		X
Mara and Sleigh 2010a														X		X			X			X
Mara and Sleigh 2010b	X															X			X			X
Munoz et al. 2010				X												X			X		X	
Oron et al. 2010		X													X	X			X			X
Ayuso-Gabella et al. 2011		X					X		X						X	X		X	X			X
Danyluk and Schaffner 2011											X						X			X		X
Drechsel and Seidu 2011		X					X					X			X	X	X		X			X
Navarro and Jimenez 2011														X		X		X	X		X	
Ottoson et al. 2011											X					X		X		X		X
Rodriguez et al. 2011											X						X			X		X
Stine et al. 2011			X									X				X[b]			X	X	X	
Ferrer et al. 2012						X		X								X			X			X
Forslund et al. 2012		X														X			X	X		X
Barker et al. 2013	X															X[d]		X	X	X		X
Ding et al. 2013										X								X		X		X
Domenech et al. 2013										X								X		X		X
Lim and Jiang 2013						X						X				X[d]			X			X
Pavione et al. 2013		X					X		X							X		X*	X			X
Puerta-Gomez et al. 2013												X					X			X		X
Seidu et al. 2013											X			X		X			X		X	

Studies marked in green include specifically leafy greens as one of the crop types under study, none of the studies focused on berries.

'*': indicates that the removal effect of the washing was included in a joint removal factor with e.g. post-harvest survival.

'[a]': used as reference pathogen for viral hazards an amalgam of RV data (dose-response including susceptibility fraction, DALY per case) and AdV data (occurrence).

'[b]': not the effect of contaminated irrigation water but effect of contaminated pesticide spray water was investigated on contamination level of fresh produce.

'[c]': Under pathogen category 'pathogenic *E. coli*' in all cases but one *E. coli* O157 was the target organism, only in Diallo et al. (2008) the pathogenic *E. coli* under study was 'diarrhea causing *E. coli*'.

'[d]': The QMRA is undertaken partially (as a scenario) for home produced vegetables (e.g. Barker-Reid et al. 2010) or performed only for home produced vegetables (e.g. Barker et al. 2013, and Lim and Jiang 2013).

6.4. RESULTS AND DISCUSSION

6.4.1. Food Science versus Water Microbiology: a different perspective

In total forty-one QMRA studies were identified for further characterization. The majority of these contained at least one stochastic variable (29/41), only twelve models were deterministic. Most QMRA studies were elaborated by a 'Water/Environmental-group' (Water perspective) (28/41), eight studies were conducted from a 'Food/Agriculture-group' (Food perspective), and five studies were done by combined research groups representing both Water Microbiology and Food Science. This is not surprising as risk assessment for water safety or wastewater reclamation has a much longer tradition compared to risk assessment for the food sector. Earlier QMRA studies considered consumption of food crops only as one of several possible exposure scenarios of (treated) wastewater, and the effect and efficiency of wastewater treatment was the main objective of those studies (e.g. in Asano et al. (1992), Tanaka et al. (1998), van Ginneken and Oron (2000)).

6.4.1.1. Variation in focus on the stage in the farm-to-fork model under consideration

For those studies written from a Water perspective (28/41), all included the Farm level as a part of the 'farm-to-fork model'; six studies included Wastewater treatment, six included the Consumer level and only two included the Processing level of fresh-cut produce, although the processing step was not an industrial process but a washing step conducted by street food venders (Seidu et al. 2008, Drechsel and Seidu 2011). Studies published by research groups with a Food perspective date back to only 2005 (Stine et al. 2005b). In the farm-to-fork continuum, most of these studies included Farm level (8/13), but compared to QMRAs performed by research groups with a Water background, the role of Packing/Processing (5/13) and the Consumer (8/13) were more often incorporated and water treatment was not included (0/13).

6.4.1.2. Variation in focus of target pathogen under consideration

The papers dealt with a wide range of pathogenic bacteria (e.g. *E. coli* O157:H7, *Salmonella* spp., *Listeria monocytogenes* and *Campylobacter* spp.), viruses (e.g. human enteric viruses, enterovirus, hepatitis A virus, norovirus and rotavirus), parasitic protozoa (e.g. *Cryptosporidium* spp., *Giardia* spp. and *Entamoeba histolytica*) and helminths (e.g. *Ascaris lumbricoides*). Articles written from a Water perspective tended to focus on human enteric viruses (20/28). It appeared that the choice of these pathogens was not driven by data availability but rather because enteric viruses are known to be highly infective, are often found in high concentrations in secondary effluent, are relatively persistent in the

environment, and are believed to be responsible for the majority of waterborne infections in developed countries such as the United States (Hamilton et al. 2006). Five of these QMRA studies elaborated from the Water perspective use so-called 'reference pathogens'. These are selected pathogens, the control of which is stringent enough to be considered adequate to control other pathogen classifications to a similar or greater extent. This concept was introduced by the World Health Organization (WHO) to aid in setting guidelines for wastewater reuse and water treatment requirements (Gibney et al. 2013). Typical reference pathogens are *Campylobacter* spp. (or *Salmonella* spp. (Drechsel and Seidu 2011)), rotaviruses (and adenovirus (NRMMC-EPHC-AHMC 2006)), and *Cryptosporidium* to model risks for bacteria, viruses, and parasitic protozoa and helminths, respectively. When QMRAs were performed for different classes of pathogens (e.g. viruses, bacteria, protozoa), the viruses most commonly presented the highest risk of infection (e.g. in Mara et al. 2007; Pavione et al. 2013; Bastos et al. 2008). All five QMRA studies focusing on *Ascaris lumbricoides* were also performed from a Water perspective. Studies from a Food or combined Food and Water perspective mostly focused on specific enteric foodborne pathogens such as *E. coli* O157:H7 (n=3/13), *Salmonella* (n=3/13) and *Listeria monocytogenes* (n=3/13). Although also viruses (n=5/13) such as hepatitis A, norovirus, and rotavirus, and parasitic protozoa (n=1/13) (*Cryptosporidium* and *Giardia*) were included as target pathogens.

6.4.1.3. Variation in focus on food crop under consideration

More than half of the forty one publications were QMRA studies concerning leafy vegetables such as salad crops, lettuce (salads) or spinach. Other commodities included bell peppers, cucumber, broccoli, cabbage, onion, kale, carrots, tomatoes, potato and cantaloupe. Leafy greens are prone to contamination with pathogens as they have large surface area (hence, greater pathogen attachment sites), are grown in close proximity to the soil, irrigated intensively and are mainly consumed raw (Melloul et al., 2001; Vega et al., 2005). Among fresh fruits and vegetables, leafy green vegetables and fresh herbs were perceived as of greatest concern in terms of microbiological hazards and received the highest priority in a joint expert meeting of FAO and WHO (FAO/WHO 2008c). This study and others (e.g. Chen et al. 2013; EFSA BIOHAZ Panel 2012b) are based on qualitative ranking of certain parameters by experts, are hence no QMRA studies and are therefore not uptaken in this review. However, they can be of interest for risk managers to set priorities in certain pathogen/commodity combinations.

6.4.2. Modeling strategies, use of assumption and surrogate data, recurring data gaps

(Quantitative) risk assessment studies have to be fit for intended purpose and demand a combination of data collection, mathematical modelling or calculations and expert insights and interpretations. Depending on the required objective and nature of available information, each assessment will result in a particular strategy or approach. So, though there is guidance on good QMRA practice, it is impossible to set a 'gold standard' for these types of studies (CAC 1999, 2007a,b). Risk assessments are data intensive and require data on a specific (usually national) context. Thus risk assessors are often confronted with lack of information and need to use surrogate data or assumptions. In the frame of the present manuscript the term 'assumption' is further defined according to the Oxford Dictionary as information which is accepted as true, *without* (experimental) proof for the specific setting. Assumptions are frequently based on expert opinion and may well lack consideration of variability. The term 'surrogate data' is used when stand-in or substituted data are based on (limited) experiments or when data obtained for another micro-organism or situation is used as a proxy for the pathogen or situation under study. Examples of surrogate data are the use of data of another micro-organism than that of concern, or another country than that of interest. In the absence of high quality data, use of surrogate data or assumptions often leads to more conservative estimates, also referred to as worst case scenarios. Constraints, uncertainties and assumptions having an impact on the risk assessment, should be explicitly considered and documented in a transparent manner (CAC 2007b).

By detailed analysis of the selected QMRA studies, surrogate data or assumptions were identified for each of the following data categories: (1) prevalence and concentration of micro-organisms of concern; (2) transmission routes (how the pathogens enter the food chain); (3) growth, removal, survival, and/or inactivation of microorganisms; (4) consumer behavior; and (5) dose-response relationship.

6.4.2.1. Filling the data gap on prevalence and concentration of pathogens in water or fresh produce along the fresh produce chain

In order to assess exposure, the prevalence and concentration of pathogens on the commodity under consideration - or further backwards in the supply chain (such as in irrigation water) - needs to be known. This was one of the major data gaps identified during this review. There are many approaches taken to overcome this data gap problem, as listed in Table 6.2.

For many reasons, there is little routine or regular monitoring of fresh produce or water for the presence of pathogens in most countries, explaining the lack of data. Even when done, pathogen prevalence is usually quite low. For example, *Salmonella* spp. prevalence reported in foods of non-animal origin as part of the European Food Safety Authority's (EFSA's) zoonoses web-based reporting from 2004 to 2011 was 0.48%. In another example, of 1860 samples of unprocessed leafy raw vegetables (from October 2006-October 2007) sampled at the entrance hall of two processing companies in the Netherlands, *Salmonella* spp. were detected in six samples (0.38% prevalence estimate) in the range of 0.019 -> 0.281 CFU per gram (Pielaat et al. 2014). Clearly, obtaining accurate data requires large sample numbers in order to construct an adequate probability distribution of pathogen concentration/prevalence for the model. Even if a large data set was collected, the sample volume and location is an important factor to be taken into account. Samples could be falsely reported as negative due to pathogen concentrations falling below assay detection limits. Indeed, factors such as assay specificity, sensitivity, and availability of internationally standardized analytical methods all affect the quality of the data obtained. In case of detection by molecular methods (e.g. for viruses), a positive test result obtained by real-time PCR does not mean that the pathogen is infectious and thus a public health hazard (Knight et al. 2013). Some studies assume that genomic copies are equal to infective pathogens (i.e. Barker et al. 2013; Ferrer et al. 2013; Lim and Jiang 2013), but this may overestimate public health risk.

To take into account the impact of positive samples having pathogen concentrations below assay detection limits, additional steps in dealing with sampling data may be imposed in some QMRAs (e.g. in Ding et al. 2013; Lim and Jiang 2013). Mota et al. (2009) applied a deterministic approach by simply performing the calculation of annual risk of infection using the limit of detection of the method involved. To consider seasonal fluctuations of pathogen loads (e.g., in reclaimed wastewater used for irrigation), it was important to sample for a prolonged period of time, comprising the whole crop cycle and growing season (e.g. in Diallo et al. 2008). Due to temperature and rainfall variations, and overflows or occasional household or industrial discharges, spatial and temporal variability are typically observed in microbial parameters of surface water (Won et al. 2013; Nnane et al. 2011). In this literature review, different approaches were identified to handle data needs related to the prevalence and concentration of microorganisms in water or fresh produce (Table 6.2.).

The *first approach* and preferred situation occurs when QMRA studies have access to relevant sampling and pathogen testing data, either data through collection by the team doing the modeling (e.g. in Ferrer et al. 2012) or through pathogen data availability from

prior representative studies i.e. same region, same type of water or food crop under consideration (e.g. in Ding et al. 2013). It is important that the suitability and robustness of the data set being used as input into QMRA is verified relative to sampling plan (number of samples and sampling locations) and analytical method performance (specificity, sensitivity and limit of detection). If situation-specific data on pathogen presence (e.g. (reclaimed) irrigation (waste)water or on produce) is missing, other strategies are used to obtain plausible estimates. As such, a *second approach* is the use of data from other production sites/countries (e.g. Lim and Jiang 2013) or from other (similar) vegetables (e.g. Carrasco et al. 2010) as surrogates for the situation under study.

A *third approach* is the use of established ratios between indicator bacteria and the specific pathogen under consideration. This strategy is used to circumvent the problem of analyzing large sample numbers or sizes for the presence of specific pathogens of low prevalence, as data for indicators are more readily available, of higher prevalence, and higher concentration. Some of the ratios most often used are described in Table 6.2. Almost all studies that use one of these ratios for estimating the concentration of enteric viruses, *Campylobacter* or *Cryptosporidium*, refer to only two limited experimental studies. The ratios for rotavirus, enterovirus and *Campylobacter* spp. are based on data from waste stabilization ponds in northeast Brazil reported by Oragui et al. (1987), the ratio for *Cryptosporidium* is based on data from ponds in Kenya reported by Grimason et al. (1993). Both studies determined the number of fecal coliforms together with the concentration of these pathogens.

The widespread application of these ratios to situations very different from those encountered in the initial data collection (i.e. raw wastewater data from tropical countries in non-epidemic situations (Mara et al. 2007) is not supported experimentally. For example, the diversity of pathogens present and concentrations in raw sewage depend upon origin of the fecal input (human/non-human sewage) (O'Toole et al. 2014) and the epidemiological status of the contributing populations (Hamilton et al. 2007), both of which differ by region. This is particularly relevant when applying these ratios to QMRA for use in developed countries (Forslund et al. 2010) or epidemic situations. The use of these ratios as proxy for other types of water such as (partially) treated wastewater (e.g. in Munoz et al. 2010) or domestic greywater (e.g. in Barker-Reid et al. 2010) is also questionable because of differences in wastewater treatment efficiency and the comparability of survival/growth of indicators versus pathogens.

Table 6.2. Data needs related to prevalence and concentration of pathogens in water or fresh produce and identified approaches (based on genuine data, surrogate (S) data or assumptions (A)) including their inherent frailty to deal with these data needs.

Data need on pathogen concentration and prevalence (including seasonal fluctuations) on fresh produce and/or in (reclaimed) water used for irrigation practices.	
(i) The use of relevant prevalence and concentration data obtained by own sampling or from prior studies being representative, i.e. same region, same type of water or food crop under consideration	e.g. Ferrer et al. (2012); Ding et al. (2013)
(ii) The use of data from another production site/countries or other similar vegetables as **surrogate** data	e.g. Barker-Reid et al. (2010); Lim and Jiang (2013); Carrasco et al. (2010) etc.
(iii) The use of **ratios between indicator bacteria and the specific pathogen** under study, based on limited experimental data, to estimate the pathogen concentration level in the initial irrigation water in a different context. E.g.:	
- Ratio enteric virus : fecal coliform in wastewater is $1:10^5$	Shuval et al. (1997); Munoz et al. (2010)
- 0.1-1 rotavirus per 10^5 *E. coli* (or fecal coliform)	Mara et al. (2007); Pavione et al. (2013)
- 0.1-1 *Campylobacter* per 10^5 *E. coli*	Mara et al. (2007); Bastos et al. (2008)
- 0.01-0.1 *Cryptosporidium* (oocyst) per 10^5 *E. coli*	Mara et al. (2007); Pavione et al. (2013)
- Others	Mara and Sleigh (2010b); Seidu et al. (2008)
When using these ratios in a different context than those observed during the experimental studies on which these are based, some **assumptions** are made:	
• The contributing source of fecal load (human/non-human) to the water is similar as in the experimental study	
• The removal efficiency of the used WWT or the survival and growth of the indicator and the pathogen are comparable.	e.g. Barker-Reid et al. (2010); Munoz et al. (2010); Drechsel and Seidu (2011)
• There is a linear relationship between the concentration of the indicator and the concentration of the pathogen of interest	e.g. Mara et al. (2007); Mara and Sleigh (2010a); Mara and Sleigh (2010b)
In order to use the ratios with the data that was at hand some indicators/microorganisms were used as **assumed surrogate** for others, e.g.:	
- *E. coli* accounts for all fecal coliforms - Enteric viruses are represented by enteroviruses } enteric virus : *E. coli* ratio is $1 : 10^5$	e.g. Barker-Reid et al. (2010)
- Data of total coliforms was used instead of fecal coliforms	Munoz et al. (2010)

	- NoV are represented by enteroviruses => 0.1 – 1 norovirus per 10^5 *E. coli* Ratios (pathogen/indicator ratios) that were initially based on occurrence in (treated) municipal wastewater were **assumed** to be applicable to calculate number of pathogens present ON produce (e.g. tomatoes, potatoes, lettuce) For other pathogen/indicator ratios there is no clear reference to experimental/screening studies E.g.: 8% of measured *E. coli* concentration is diarrhegenic/pathogenic or a pathogen/indicator ratio was assumed: e.g. ratio of *E. coli* O157:H7 to *E. coli* is $1:10^6$.	Mara and Sleigh (2010b) Seidu et al. (2008); Forslund et al. (2010); Forslund et al. (2012) Diallo et al. (2008) Seidu et al. (2013)
(iv)	The fecal loading approach to estimate the concentration of a specific pathogen in water.	Barker et al. (2013); Ottoson et al. (2011)
(v)	The initial contamination level of the (initially untreated) irrigation water or produce was simulated by different potential contamination levels, <u>or</u> the use of one assumed scenario.	e.g. Rodriguez et al. (2011); Domenech et al. (2013); Mara et al. (2007); Bastos et al. (2008) <u>Or</u> Van Ginneken and Oron (2000); Oron et al. (2010)

The case for enteric viruses is a good one. For example, there is ample evidence that enteric viruses may persist after water disinfection treatments that eliminate bacteria (Simmons and Xagoraraki 2011; Ottoson et al. 2006; Rodriguez-Manzano et al. 2012) and in general bacteria are poor indicators of the presence of viruses and parasitic protozoa (Rimhanen-Finne et al. 2004; Agullo-Barcelo et al. 2013; Jurzik et al. 2010; He et al. 2012). As such, the existence of a linear relationship between the concentration of an indicator bacteria and e.g. a specific enteric virus is highly unlikely, particularly a pathogen with a distinctive seasonality and prevalence. The lack of a fixed correlation between pathogen and (a single) bacterial indicator, and hence the invalidity of these ratios, have been highlighted by Silverman et al. (2013) and Cutolo et al. (2012). For example, the inutility of using *E. coli* results to model virus health risks associated with the reuse of domestic greywater has been recently demonstrated in a study by O'Toole et al. (2012), with a finding of no statistical correlation between the presence of the indicator and viruses.

Another point in the use of these ratios is that assumptions have been suggested to alter these ratios: the original data in Oragui et al. (1987) and Grimason et al. (1993) comprised fecal coliforms and several studies assumed to replace data of fecal coliforms with data of *E. coli* (e.g. in Barker-Reid et al. 2010; Mara and Sleigh 2010b), or total coliforms (e.g. in Munoz et al. 2010). In Mara and Sleigh (2010b) it was assumed that noroviruses could be represented by enterovirus concentrations (Table 6.2.), despite the distinct variability in seasonality of human NoV and enteroviruses. Since in regions in the temperate northern hemisphere, where most data is available, infections due to enteroviruses generally reach a peak in late spring and early summer (Costan-Longares et al. 2008) or summer and early fall (Sedmak et al. 2003; Skraber, S. et al. 2004), while on the contrary NoV infections mainly occur during the cooler winter months (Ahmed et al. 2013, de la Noue et al. 2014). These pathogen/indicator ratios have also been used to estimate pathogen contamination of fresh produce. In studies of Forslund et al. (2010), Forslund et al. (2012) and Seidu et al. (2008), the ratio was used to estimate rotavirus concentration on potatoes, tomatoes and lettuce respectively, that were all irrigated with (treated) wastewater. The validity of this practice is questionable, especially in case of Seidu et al. (2008), since the use of poorly treated poultry manure and cow dung as fertilizer is common practice in Ghana. These non-human waste materials would not be expected to harbor enteric viruses and use of these ratios under these circumstances could lead to an overestimation of rotavirus concentration on produce. Taken together, extrapolation of relationships found in a specific wastewater system to other regions, other water sources and other matrices (e.g. on fresh produce) should be approached cautiously. Consequently, critical evaluation of QMRA outcomes that result when using these ratios is necessary and can help identify faulty

assumptions. This was observed by Barker-Reid et al. (2010), who found overestimation of enteric virus risk associated with consumption of brassicaceous vegetables that were irrigated with greywater derived from kitchens. This higher risk was a consequence of the use of fecal coliform indicator ratios as proxy for enteric viruses, with elevated levels of the former likely associated with a non-human source of fecal contamination, perhaps from the washing of chicken carcasses.

A *fourth strategy* to address the lack of data on pathogen prevalence and concentration is the fecal loading approach. This resembles the previous approach as it likewise circumvents the constraints of analyzing large sample sizes of water for the presence of a specific pathogen. However, in contrast to the previous approach, it does not require extrapolation of experimentally determined ratios, but relies on the use of a reasoned calculation. This rationale requires first (indirectly) the fecal loading of the potential irrigation water by e.g. use of a determined amount of *E. coli* per g (human) feces (Barker et al. 2013) in greywater or by assuming that all *E. coli* in river water is originating from feces from herds harbouring zoonotic pathogens such as verocytotoxin producing *E. coli* (VTEC) as mentioned by Ottoson et al. (2011). Next, a known pathogen shedding concentration in feces (e.g. number of NoV particles/g feces in Barker et al. (2013)) or a known pathogen to *E. coli* ratio in feces (e.g. VTEC/*E. coli* in infected herd and the proportion of infected herds) is needed. Thus the NoV concentration in domestic greywater in Australia (Barker et al. 2013) and the VTEC concentration in surface water contaminated by cattle herds in Sweden (Ottoson et al. 2011) could be calculated. A downside of this approach is the need for many input data for the construction of the exposure model, which likewise increases the complexity and may introduce greater uncertainty in the final estimate of pathogen concentrations (Mok et al. 2014). The latter was observed during sensitivity analysis for both studies (Barker et al., 2013; Ottoson et al., 2011), as the norovirus shedding rate and the ratio VTEC/*E. coli* ratio in manure were responsible for the majority of the variability in probability of infection or illness.

A final (*fifth*) *option* to deal with lack of data is to simulate the initial contamination and/or prevalence of the pathogen for irrigation water/incoming product by the use of different potential scenarios (e.g. in Danyluk and Schaffner 2011) or the use of one assumed scenario (e.g. in van Ginneken and Oron 2000; Oron et al. 2010) (Table 6.2.). In some QMRA studies, different (potential/existing) water/produce quality guidelines concerning maximum concentration of *E. coli* are selected in order to verify the validity of these guidelines to reduce the risk of defined pathogen exposure. Hence, the conversion to pathogen concentration is done according to the third approach using ratios or by the fourth approach using fecal loading. In some studies, scenarios using different pathogen

concentrations in irrigation water were simulated in order to obtain maximum tolerable estimates of pathogens that would comply with certain acceptable maximum risk levels (e.g. in Navarro and Jiménez 2011; Mara et al. 2007).

6.4.2.2. Filling the data gap regarding pathogen transfer during irrigation and washing of fresh and fresh-cut produce

TRANSFER FROM IRRIGATION WATER TO THE CROP

In simulating contamination routes, data necessary to model the transfer of pathogens from the (production) environment to fresh produce are needed. Several factors can contribute to the likelihood and degree of pathogen contamination to fresh produce at this phase, including water used for irrigation or pesticide/fungicide treatment; soil and insufficiently composted manure or biosolids used as fertilizers; wild or neighboring domestic animals or livestock grazing on adjacent fields (and associated run-off water); harvest or washing equipment; and field workers (Liu et al. 2013; Ilic et al. 2012; Olaimat and Holley 2012). Of these, contaminated irrigation water has received the greatest attention. However, food handlers, particularly for hand-picked products, have been identified as important contributors to the overall microbiological quality of fresh produce (Leon-Felix et al. 2010). So has contamination with pathogens present in soil/biosolids (Seidu et al. 2008). The latter is particularly relevant to developing countries, in which poorly treated manure or biosolids/sludge are still used as fertilizer. These two risk factors (food handlers and biosolids) should ideally be included as a source of contamination in fresh produce production. However the present review focuses on water as a contamination route and therefore only the approaches taken and assumptions made for modeling transfer from irrigation and washing water to the food crop were analyzed (Table 6.3.) and further discussed.

Different strategies have been used to model or estimate the number of pathogens on (the surface of) the crop after irrigation. *A first strategy* is to use surrogate data to estimate the amount of water clinging to the crop after irrigation and assuming that any microorganisms contained in the residual water remaining on the edible product would cling to the vegetables, also after evaporation of the water. The degree of contamination can be estimated using this approach if the microbial load of the water is known and if an estimate is at hand of the amount of irrigation water retained by the produce (van Ginneken and Oron 2000; Oron et al. 2010). Often, estimates of the amount of water retained on the produce are based on surrogate data originating from one single study, i.e. Shuval et al. (1997), in which the worst-case scenario was simulated by total immersion of pre-weighed cucumber (n=26) and lettuce (n=12) in water. These results (i.e. lettuce: on average 10.8 ml/100g; cucumber: on average 0.36 ml/100g), which were originally presented as point-

estimates, have been commonly used in studies that assessed the transfer of pathogens by spray irrigation (Lim and Jiang 2013). Hamilton et al. (2006a) imposed a normal distribution on these data (used also in e.g. Barker et al. 2013; Ottoson et al. 2011). In addition, Hamilton et al. (2006a) determined the amount of water retained on some other vegetable products. In this study, the amount of water retained on broccoli (n=100) and three cultivars of cabbage (3 x n=20) was determined in field tests using overhead irrigation. The resulting distributions have served as input data for other QMRA studies (e.g. in Hamilton et al. 2006b; Barker-Reid et al. 2010). These limited data for lettuce, cucumber, broccoli and cabbage have been used as stand-in data for other vegetables (e.g. in Munoz et al. 2010; Mota et al. 2009). When different crops are compared, the risks for lettuce tend to be higher due to this product's relatively higher water retention rate (Hamilton et al. 2006a; Lim and Jiang 2013).

In the absence of data, a *second approach* is to use assumptions to estimate the amount of water adhering to crops after irrigation. This was done in two of the oldest QMRAs included in this review (assuming an average daily exposure of 10 ml water) (i.e. Asano et al. 1992; Tanaka et al. 1998). Some studies have also used this approach to estimate the microbial load of root vegetables such as onions (Mara et al. 2007) and carrots (Mara and Sleigh 2010a). In studies by van Ginneken and Oron (2000) and Oron et al. (2010), assumptions were made on the amount of water retained on the crop (fruits and vegetables), differentiating between spray irrigation, drip irrigation and subsurface drip irrigation (Table 6.3.).

A third approach to fill the data gaps about pathogen transfer from water to produce has been to use data from field experiments in which the produce had been irrigated during growth with the relevant type of irrigation water under consideration (with naturally occurring micro-organisms). The subsequent microbial load of the irrigated crops is then determined upon harvest and used as input data in the QMRA model. The first study taking this approach was that of Seidu et al. (2008), who used data from previous studies by Obuobie et al. (2006) and Amoah et al. (2007a,b). These studies assessed the concentration of *Ascaris* and fecal coliforms on lettuce irrigated with different water types (drain, stream and piped water). For the QMRA of Finley et al. (2009), field data on the contamination level of lettuce, carrots and peppers irrigated with (treated) greywater or tap water at soil-level was obtained for fecal coliforms and fecal streptococci. In Forslund et al. (2010; 2012) field studies were performed to assess the contamination of potatoes and tomatoes with *E. coli* after the use of different treated waters and irrigation methods. Note that in all of these field studies, except for the detection of *Ascaris*, fecal indicator bacteria were monitored, after which in all but one study (i.e. Finley et al. 2009), a pathogen/indicator

ratio was used to estimate the amount of rotaviruses on the crop. As such, the fecal indicator bacteria data were used as proxy for the transfer and attachment of viruses.

The last and *fourth approach* is also based on field data. In this case, field trials were performed not simply to use the data, but rather to simulate the transfer of the pathogens in (irrigation) water to the crop via the development of a formula to calculate transfer rate. In two studies done by Stine et al. (2005b; 2011), field trials were conducted to obtain the percentages of micro-organisms transferred from water to the surface of fresh produce via irrigation and by application of water-diluted pesticide spray, respectively. In both studies the coliphage PRD1 and *E. coli* ATCC 25922 were used as surrogates for the transfer of HAV and *Salmonella*, respectively. Two different irrigation methods (subsurface drip irrigation and furrow irrigation) were evaluated in the field studies and both trials were performed on three produce types: cantaloupe, iceberg lettuce and bell peppers. Bastos et al. (2008) performed field trials to obtain formulae for low-growing crops and high-growing crops that related the concentration of *E. coli*/100 ml irrigation water with the *E. coli* concentration per gram on the crop. In this case, watering was done using cans and *E. coli* was used as a surrogate for *Giardia*, *Cryptosporidium*, rotavirus and *Campylobacter*. These data were later also used by Pavione et al. (2013).

The latter two approaches (i.e., using data from field trials), have an advantage in that the effect of repeated irrigation with contaminated water and attachment, survival and growth of the surrogate organisms during production, are included in the estimates. In contrast, the first two approaches only take into account the amount of water clinging to the crop after one irrigation event (or water submersion) after which survival is included during the subsequent withholding period (period between last irrigation and harvest). However, a downside of the latter two approaches is that the data may not be very applicable to other situations, as different environmental and climatic conditions could influence the final microbial loads. In several field studies, the influence of crop type was investigated by e.g. selecting (a root), leaf, and fruit crop each of varying heights (Finley et al. 2009; Bastos et al. 2008; Stine et al. 2011; Stine et al. 2005b).

TRANSFER OF MICRO-ORGANISMS / CROSS-CONTAMINATION DURING POST-HARVEST RINSING AND WASHING

Contamination of produce due to cross-contamination during processing/packing (e.g. washing) has also been modeled, albeit in a limited number of studies (e.g. Danyluk and Schaffner 2011). There is increasing interest in this phenomenon due to the rising market for pre-packaged, washed salad vegetables. Validation studies to characterize transfer/cross-contamination rates during industrial processing or salad preparation has been identified as a data gap (Danyluk and Schaffner 2011; Rodriguez et al. 2011; Puerta-

Gomez et al. 2013). Recent studies evaluated cross-contamination from nylon brushes and peelers that were contaminated with viruses to uncontaminated carrots and celery (Wang et al. 2013), and in Chapter 5 cross-contamination of lettuce with bacteria (*E. coli*) and viruses (MS2 and MNV-1) during simulation of industrial washing procedures of fresh-cut lettuce washing was modeled.

Table 6.3. Data needs identified to model the transmission routes describing how pathogens are transferred from environment (water) to the crop and identified approaches (based on genuine data, surrogate (S) data or assumptions (A)) to deal with these data needs.

Knowledge on the transfer of pathogens during irrigation on farm level and effect of different irrigation strategies on contamination level of fresh produce.	
(i) Use of **surrogate data** (based on limited experiments) to estimate the amount of water clinging to the crop after spray irrigation (V_{prod}), data can be based on:	
- <u>submersion experiments</u>:	
• Shuval et al. (1997): Lettuce: 10.8 ml/100 g; cucumber: 0.36 ml/100 g	Shuval et al. (1997); Petterson et al. (2001); Mota et al. (2009)
Variants on this base data have been applied:	
➔ Lettuce: Normal, μ=0.108, σ=0.019 (truncated at 0) (ml/g)	Hamilton et al. (2006a); Hamilton et al. (2006b); Barker et al. (2013); Ottoson et al. (2011); Ayuso-Gabella et al. (2011)
Uniform (8.9, 12.7) (ml/100 g)	Lim and Jiang (2013)
Range: 10-15 ml/100 g	Mara et al. (2007); Mara and Sleigh (2010b); Diallo et al. (2008)
Range: 10.8-15 ml/100 g	Drechsel and Seidu (2011)
V_{prod} = 5 ml on 40 g of lettuce	NRMMC-EPHC-AHMC (2006)
➔ Cucumber: Normal, μ=0.0036, σ=0.0012 (truncated at 0) (ml/g)	Hamilton et al. (2006a); Hamilton et al. (2006b)
Uniform (0.24, 0.48) (ml/100 g)	Lim and Jiang (2013)
• Bartz (1988) : tomato: 0.04-1.66 ml/100 g -> Uniform (0.04-1.63) ml/100 g	Lim and Jiang (2013)
- <u>Spray-irrigation experiments</u> => Hamilton et al. (2006a):	
• broccoli (n=100): Log Logistic, α=4.246, $\beta=1.583\times10^{-2}$, $\lambda=1.085\times10^{-3}$ (μ=0.0185)	Hamilton et al. (2006a); Hamilton et al. (2006b)
• Savoy King/Grand Slam cabbage (2 x n=20): Empirical CDF (μ=0.0352) (ml/g)	Hamilton et al. (2006a); Hamilton et al. (2006b)
• Winter head cabbage (n=20): Empirical CDF (μ=0.0889) (ml/g)	Hamilton et al. (2006a); Hamilton et al. (2006b)

	When no specific surrogate data was available, some studies used data as stated above as **surrogates** for other types of vegetables and fruits. E.g. - The use of remaining water volume on cucumber, for other 'smooth produce' such as tomatoes and bell peppers - The use of remaining water volume on lettuce as surrogate for other leafy vegetables, and remaining volume of water on cucumber as surrogate for other vegetables and fruit - The use of V_{prod} for Winter head cabbage as surrogate for all brassicaceous vegetables This approach has as inherent **assumption** that any microorganisms contained in the residual wastewater/irrigation water remaining on the irrigated vegetables would cling to the vegetables even after the wastewater itself evaporated.	Mota et al. (2009) Munoz et al. (2010) Barker-Reid et al. (2010)
(ii)	If no relevant surrogate data is available, **assumptions** have been used for the amount of water clinging to crops after irrigation. E.g. • Assuming an average daily dose of 10 ml when eating vegetables • Assuming that the volume of irrigation water remaining on onion is 1-5 ml per 100 g onions • Assuming 3-5 ml of irrigation water remaining on 100 g carrots • In van Ginneken and Oron (2000) assumptions have been made on the amount of water remaining on fruits and vegetables when three different irrigation techniques were used: - Spray irrigation: 16 ml/100 g - Onsurface drip irrigation: ~triangular distribution (min.: 0.016 ml/100 g; max.: 1.6 ml/100 g; mean: 0.16 ml/100 g of plant matter) - Subsurface drip irrigation: ~triangular distribution (min.: 0.0016 ml/100 g; max.: 0.16 ml/100 g; mean: 0.016 ml/100 g)	Asano et al. (1992); Tanaka et al. (1998) Mara et al. (2007) Mara et al. (2010a) Van Ginneken and Oron (2000) Van Ginneken and Oron (2000); Oron et al. (2010) Van Ginneken and Oron (2000); Oron et al. (2010)
(iii)	The effect of the relevant type of irrigation water under study on the contamination level of fresh produce was examined during a *field experiment*. The level of microbial contamination of the crops (mostly fecal indicators) under study were determined upon harvest and used as input data of the QMRA model.	Seidu et al. (2008); Finley et al. (2009); Forslund et al. (2010); Forslund et al. (2012)

(iv)	The performance of field trials using surrogate microorganisms to **model** the transfer of specific pathogens in irrigation/pesticide water to the specific crop. Surrogates that are used:	
	- Coliphage PRD and *E. coli* ATCC 25922 as surrogate for the transfer of HAV and *Salmonella*, respectively	Stine et al. (2005b); Stine et al. (2011)
	- *E. coli* as surrogate for the transfer of *Giardia*, *Cryptosporidium*, rotavirus and *Campylobacter*.	Bastos et al. (2008); Pavione et al. (2013)
Additional studies on the effect of different irrigation water strategies on the pathogen load of different crops.		
(additional validation) studies on occurrence and effect of cross-contamination during farm to fork chain (e.g. washing, cutting, packaging). Including data on distribution of cross-contaminated microorganisms in processed produce.		
(i)	The use of data based on experiments to simulate the transfer of the pathogen to the crop. Some studies use data obtained using surrogate microorganisms others data based on cross-contamination studies that used the relevant pathogen under study. E.g.	Ding et al. (2013). Rodriguez et al.(2011)
	- The use of nonpathogenic surrogates (PRD1 phage and *E. coli*) instead of the pathogens under study (HAV and *Salmonella*) to determine the fraction of microorganisms present on surface of cantaloupe that are recovered from the flesh after cutting the cantaloupe.	Stine et al. (2005b); Stine et al. (2011)
	- Danyluk and Schaffner (2011) modeled the cross-contamination of leafy greens with *E. coli* O157:H7 due to washing at processing stage using data of Zhang et al. (2009) on the relevant pathogen under study.	Danyluk and Schaffner (2011)
(ii)	When no relevant data are at hand, the effect of cross-contamination can be simulated using different cross-contamination scenarios (with e.g. different types of distribution of bacterial load and different cross-contamination levels).	Puerta-Gomez et al. (2013)
(iii)	Assuming that no cross-contamination of fruits and vegetables after harvest is occurring and therefore contamination of the crops is solely due to farm level contaminations	e.g. Oron et al. (2010); Al-Juaidi et al. (2010)

6.4.2.3. Filling the data gaps on reduction/growth/survival of microorganisms along the fresh produce production chain

GROWTH AND SURVIVAL

Due to the frequent unavailability of relevant pathogen survival or persistence data on fresh produce, surrogate data and assumptions have mostly been used in selected QMRAs. However, the preferred *(first) approach* is the use of actual growth curves or studies on persistence obtained for the relevant pathogen on the specific produce item under consideration, e.g. Stine et al. (2005b; 2011). Yet in some cases the use of surrogate data or surrogates *(second approach)* is more practical or even a necessity. The latter is the case for example for the non-cultivable human norovirus (Knight et al. 2013). Petterson et al. (2001a), for example, used *Bacteroides fragilis* bacteriophage B40-8 as a surrogate for human enteroviruses to estimate their persistence on lettuce. *B. fragilis* phage B40-8 was chosen because it is considered as a conservative model for human enteric viruses and may be expected to be inactivated at a slower rate than the human viruses (Petterson et al. 2001b). The resulting first order decay constant ($k= 1.07d^{-1}$, $\sigma= 0.07$) has been used in several other studies (e.g. Al-Juaidi et al. 2010), including some that used this model to represent persistence of enteric viruses on other types of produce (Hamilton et al. 2006a; 2006b). Decay or the loss of viability/infectivity of pathogenic microorganisms is traditionally modeled assuming a simple first-order kinetic model, where the decay constant is affected by various environmental factors (e.g., temperature, solar radiation, relative humidity and presence of inhibiting/inactivating substances). However, simple, first-order (single-phase) die-off is probably not accurate as most soil and subsurface environments are highly heterogeneous and because of the potential for long-term survival of persistent subpopulations and/or re-growth in the environment (Bradford et al. 2013). As such, biphasic survival kinetics have been observed in both water (e.g. Easton et al. 2005; Ahmed et al. 2014) and fresh produce production environments (e.g. Seidu et al. 2013; Petterson et al. 2001b). In biphasic decay kinetics an initial rapid decay is noted, often followed by an attenuated, slower decay. The inclusion of the possibility of a biphasic decay in QMRA is important as the survival of pathogens, and hence the predicted infection risk, can be significantly underestimated if the presence of a persistent sub-population of the microorganisms is not considered (Petterson and Ashbolt 2001; Seidu et al. 2013).

Table 6.4. Data needs related to growth/survival/removal/inactivation of microorganisms along the fresh produce chain and identified approaches (using genuine data, surrogate (S) data or assumptions (A)) to deal with these data needs.

Specific survival/growth data for very specific situation (e.g. survival in pesticide spray, crop specific survival) or for the pathogen under study is often missing	
(i) The use of growth and/or survival data obtained for the relevant pathogen on the specific crop under study, ideally when simulating relevant environmental conditions.	e.g. Stine et al. (2005b); Stine et al. (2011); Ding et al. (2013); Puerta-Gomez et al. (2013)
(ii) The use of data based on experiments in which a **surrogate** microorganism was used instead of the specific pathogen under study or the use of data based on experiments performed on a different type of produce then the produce under study, to study survival/growth of a specific pathogen on a specific crop. E.g.:	
- use of *Bacterioides fragilis* bacteriophage B40-8 as a surrogate for enteroviruses or other enteric viruses	e.g. Petterson et al. (2001a); Al-Juaidi et al. (2010); Barker et al. (2013)
- use of survival data obtained for lettuce for another produce: e.g. cucumber, broccoli and cabbage	e.g. Hamilton et al. (2006a); Hamilton et al. (2006b);
Models are often constructed assuming a simple first-order kinetic model.	
(iii) The use of **estimates** or **assumptions** on log reductions of the pathogen on the crop at pre-harvest and/or post-harvest stage.	
- Estimates for log reductions comprising survival on field but also removal/inactivation steps further in the chain (e.g. washing by consumer) e.g.:	
• Assumption of total virus inactivation/removal of 3 or 2-3 logs as rough, conservative estimation for inactivation on field and/or as estimate for removal by e.g. washing and survival post-harvest	e.g. Shuval et al. (1997); Mara et al. (2007); Seidu et al. (2008); Pavione et al. (2013)
- Assumptions, e.g.:	
• Assumption of a first order decay with as decay constant $k = 0.69\ d^{-1}$ for enteric viruses	e.g. Asano et al. (1992); Tanaka et al. (1998); Munoz et al. (2010); van Ginneken and Oron (2000); Oron et al. (2010); Hamilton et al. (2006a)

(iv)	**Assuming** pre- and/or post-harvest decay or effect of storage to be negligible relative to the shelf life.	e.g. Bastos et al. (2008); Mota et al. (2009); Barker-Reid et al. (2010); Mara and Sleigh (2010a); Ottoson et al. (2011); Stine et al. (2011)
Specific experimental data on the removal/inactivation of the pathogen		
(i)	The use of removal/inactivation data obtained for the relevant pathogen on the specific crop under study.	e.g. Puerta-Gomez et al. (2013); Doménech et al. (2013)
(ii)	The use of data based on experiments in which a **surrogate** microorganism was used instead of the pathogen under study or which was performed on a different type of produce to study the effect of removal and/or inactivation strategies (e.g. washing) of a specific pathogen on a specific crop.	e.g. Barker et al. (2013); Carrasco et al. (2010);
(iii)	The use of **estimates** or **assumptions** on log reductions of the pathogen on the crop (the same as for survival/growth). E.g.:	
	- Assumption of total virus inactivation/removal of 3 or 2-3 logs as rough, conservative estimation for inactivation on field and/or as estimate for removal by e.g. washing and survival post-harvest	e.g. Shuval et al. (1997); Pavione et al. (2013)
	- Assuming that the combined effect of washing (1 $\log_{10}$ reduction (WHO (2006)) and disinfection (2 $\log_{10}$ reduction (WHO (2006))) during salad preparation would lead to a 3 $\log_{10}$ reduction of rotavirus.	Seidu et al. (2008)
(iv)	**Assuming** that post-harvest removal/inactivation is negligible (worst-case scenario).	e.g. Mota et al. (2009); Barker-Reid et al. (2010); Bastos et al. (2008); Forslund et al. (2012)

A *third approach* is the use of estimates or assumptions on log reductions of the pathogen on the crop/plant that takes place during plant growth in the field or post-harvest. Some studies have used estimates for log reductions, whether or not combined with removal by e.g. washing/disinfection (during consumer preparation) (e.g. used by Shuval et al. 1997; Pavione et al. 2013). An assumption that has been frequently used is that for the persistence of enteric viruses, a first order decay as a function of time ($\mu_1 = \mu_0 * e^{(-kt)}$) is appropriate, with a generic decay constant k of 0.69 d^{-1} (e.g. Asano et al. 1992; Munoz et al. 2010). This constant is primarily used to model decay during the withholding period in the field. This same decay constant was also used in an early risk assessment dealing with the decay of viruses in an Illinois river (Haas 1983). But after sensitivity analysis, Haas (1983) concluded that variation in this decay constant resulted in the greatest variation in the resulting risk estimate and hence particular attention should be given to obtain data on viral decay in order to develop more precise estimates of risk. A similar conclusion about the importance of the selected decay model/constant was drawn by Petterson et al. (2001a), Hamilton et al. (2006a) and Seidu et al. (2013).

As a last *(fourth) approach*, some studies assumed pre- and/or post-harvest decay to be negligible (e.g. Mota et al. 2009; Hamilton et al. 2006a). However, many of these QMRA articles relate to viruses, and viruses are inert and relatively stable under common (assumed) storage conditions of fresh produce. However, enteric bacterial pathogens such as *Salmonella* and pathogenic *E. coli* may have the ability to multiply (or die) on fresh-cut produce, depending upon storage conditions. As there is an increasing trend towards buying pre-packed leafy greens with shelf lives up to two weeks or longer, parameters such as microbial survival and growth throughout the farm-to-fork continuum should be taken into account (Ottoson et al. 2011). Some studies did include survival/growth during storage at retail, in food service operations or at home (e.g. Carrasco et al. 2010; Puerta-Gomez et al. 2013).

REMOVAL AND INACTIVATION

Consideration of post-harvest inactivation and removal strategies such as washing (with or without the use of sanitizers in the water), irradiation or peeling were often absent in QMRA studies, predominantly because half of the studies just did not include further processing or consumer preparation in the model. A few studies (n=9) considered washing at the consumer phase (e.g. Ottoson et al. 2011; Barker et al. 2013) and in some cases (n=6), at the processing level (e.g. Rodriguez et al. 2011; Carrasco et al. 2010). Again, different approaches can be taken for modeling pathogen removal/inactivation during washing or decontamination (Table 6.4.). Similar to approaches described above, some QMRA studies use rough estimates of the reduction of pathogens by washing, often in

combination with survival (e.g. Shuval et al. 1997; Pavione et al. 2013). Other studies have used crop specific experimental data on the effect of washing on the produce and pathogen(s) under consideration (e.g. Puerta-Gomez et al. 2013; Doménech et al. 2013). The use of removal/inactivation data derived from experimental studies on other types of crops as proxy for a different product has been applied, e.g. shift from Brussels sprouts to lettuce (Carrasco et al. 2010). For other reasons, the use of surrogate data is sometimes a necessity, as is the case for non-cultivable pathogens like noroviruses (e.g. in Barker et al. 2013).

Next to washing and/or disinfection, irradiation was also investigated as an inactivation strategy for *Escherichia coli* O157:H7 and *Salmonella* spp. on fresh-cut bagged lettuce (Rodriguez et al. 2011) and ready-to-eat baby spinach (Puerta-Gomez et al. 2013), respectively. A crude estimation of pathogen reduction achieved by peeling has been considered in the case of carrots (e.g. Mara and Sleigh 2010a).

In general, pathogen growth, survival/persistence, removal and inactivation data and predictive models have been identified as a data gap for QMRA, both pre- and post-harvest (Table 6.4.). In the pre-harvest phase, climatic conditions (such as temperature, solar radiation or relative humidity) can influence the survival of pathogens in the field (Petterson et al. 2001b). Survival of microorganisms has also been suggested to be crop specific (Verhaelen et al. 2012; Macarisin et al. 2013) and could be affected by the competing microbiota present, which is impacted by many factors (Ottoson et al. 2011), and internalization. Moreover, post-harvest decay or growth along the farm-to-fork continuum is not always included in QMRA studies and can be relevant, in particular for bacterial pathogens, if longer shelf lives are applied. Knowledge on survival and growth of pathogens on specific fresh produce commodities is accumulating and data on the use and performance of sanitizers to avoid cross-contamination during washing and decontamination of fresh(-cut) produce is becoming increasingly available. Some examples include Mansur et al. (2014), who produced a growth model for *E. coli* O157:H7 on treated kale; Carratala et al. (2013b), who described persistence of human adenoviruses (hAdV) in water under different environmental conditions; Zeng et al. (2014) who described growth of *E. coli* O157:H7 and *Listeria monocytogenes* in packaged fresh-cut Romaine mix at fluctuating temperatures anticipated during commercial transport, retail storage, and display; and Bozkurt et al. (2014), who modeled thermal inactivation of human norovirus surrogates in spinach. Again, use of surrogates requires caution. Some surrogates commonly used for norovirus, such as feline calicivirus strain F-9, have been shown to be less tolerant to chlorine treatment and thermal processing (Nowak et al. 2011; Topping et al. 2009). In the discussion of growth, survival, removal and inactivation the possible

effect of internalization of pathogens inside the plant tissue can also be considered as a data gap and could be relevant to consider (Sales-Ortells et al. 2014). Internalization of human pathogens can occur through root uptake, and through cellular structures (e.g. stomata) or wounds. This physical entrapment below the surface could function as protective shelters making post-harvest treatments such as chlorine sprays and washes ineffective (Hirneisen et al. 2012; Hirneisen and Kniel 2013b).

6.4.2.4. Filling the data gap on consumer behavior

Consumer behavior, both practices and consumption patterns, influences exposure and hence risk (CAC 1999). Relevant fresh produce consumption data, including frequency and portion size for key populations, are essential for exposure assessment (Le Donne et al. 2011; Hoelzer et al. 2012). Such data have been used in QMRA studies (e.g. by Ferrer et al. 2012) (Table 6.5.). However, relevant national consumption data are not always available for every country and hence data derived from other countries and/or populations is frequently used as proxy (e.g. in Navarro and Jimenez 2011; Barker et al. 2013) (Table 6.5.). Frequently used consumption data are those derived from the US National Health and Nutrition Examination Survey (NHANES) further elaborated by US Environmental Protection Agency (e.g. in Navarro et al. 2009; Oron et al. 2010) while an European database is not yet available and still national consumption surveys are applied (Jacxsens et al. submitted). Consumption data derived from official institutes such as US NHANES, US EPA or EFSA are often expressed as daily consumption (g/day), mostly to be used for nutritional purposes or risk assessments associated with chronic chemical exposures studies (e.g. Vinci et al. 2012; De Boevre et al. 2013). Of course, in QMRA in which acute exposure is the problem, average consumption over time is less relevant than the portion and frequency of consumption of a product (CAC 1999). It should also be noted that it is difficult to compare consumption data from different countries because of different data collection methods and resources that can go into such data collection (Le Donne et al. 2011; EFSA 2013). Consequently, dietary surveys can differ with respect to a number of parameters affecting the level of detail and the accuracy of the collected data, such as: (i) the dietary assessment method e.g. 24h recalls, food frequency questionnaires (FFQ) or via diaries; (ii) the number of days over which information is collected; (iii) sampling design; and (iv) method for quantification of portion sizes. In an effort to provide more standardization, European countries are engaged in an effort to harmonize collection of consumption data among countries (EU project EU Menu http://www.efsa.europa.eu/en/datexfoodcdb/datexeumenu.htm). In the USA, a comprehensive study to obtain harmonized data on fresh produce consumption was completed (Hoelzer et al. 2012). Specific populations such as children and elderly can be

particularly vulnerable to certain microbes and this should also be considered when using consumption data (Kroes et al. 2002; EFSA 2009). Another approach for dealing with consumption data needs is to use assumptions about portion size (e.g. Petterson et al. 2001a; Ottoson et al. 2011) and/or consumption frequency (e.g. in Shuval et al. 1997).

For instance in studies where actual risk estimates are not required, e.g. methodological studies (e.g. Petterson et al. 2001a; Petterson and Ashbolt 2001) and studies whose main objective is to analyze the effect of different scenarios (e.g. different risk mitigation strategies) (e.g. Ottoson et al. 2011, Carrasco et al. 2010), the use of surrogate data or assumptions on consumption are acceptable. However when an actual risk estimate is the objective, or when exposure to different crops is compared, relevant consumption data is a prerequisite. Indeed, it has been shown in sensitivity analysis that the amount of produce consumed or serving size can have an important effect on the uncertainty surrounding a risk estimate (Hamilton et al. 2006a; Carrasco et al. 2010, Lim and Jiang 2013).

Consumption habits can be highly culturally dependent, e.g. a serving size of 85 g of cut leafy greens was used in a QMRA (Danyluk and Schaffner 2011) as a representative portion size for the USA, while a consumption portion of 10-12 g was used to model risks associated with raw salads that are mainly sold as 'street-food', in Ghana (Seidu et al. 2008). Mara and Sleigh (2010b) compared the effects of two different consumption patterns (100 g every two days vs. 10-12 g on each of four days a week) on norovirus log reduction needed to comply with a tolerable level of risk associated with consumption of waste-water irrigated lettuce. A one log difference was observed in pathogen reduction required along the farm-to-fork chain, depending upon the consumption pattern chosen for the risk modeling. Clearly, the application of one countries' consumption data to another may not always be relevant. Another standard assumption made in QMRA is that all of the commodity consumed is produced under the conditions being modeled (e.g. use of recycled water or wastewater at farm level; occurrence of washing at the processing stage) (NRMMC-EPHC-AHMC 2006; Pavione et al. 2013). This is necessary to investigate the possible impact of defined risk mitigation strategies when compliance is 100%, but it does produce a worst-case scenario risk estimate.

Another limitation of most consumption data is the absence of information on the state of a food item at consumption (e.g. raw, washed, peeled, cooked, stir fried, steamed) (Agudo et al. 2002; EFSA 2013; Soerjomataram et al. 2010). The state of the product can be highly relevant to the actual risk estimation as several consumer practices can have major influences on the microbial characteristics of the product at the time of consumption. Such preferences can be culturally dependent, requiring region-specific data on household practices such as frequency or rigor of vegetable preparation/washing (Barker-Reid et al.

2010; Pavione et al. 2013) and the proportion of fruits and vegetables eaten raw. Such data are lacking for use in most QMRA studies. In the absence of more specific data on consumer behavior, the most frequently used assumption for QMRA is that the produce item(s) under study are all eaten raw (e.g. lettuce, cucumber, but also broccoli or cabbage) (e.g. in Hamilton et al. 2006a). Studies can circumvent this problem by using consumption data specific to raw produce (e.g. Carrasco et al. 2010), and for some vegetables, such as lettuce, it is reasonable to assume that most product will be consumed raw. For other vegetables such as broccoli, cabbage, spinach or carrots, the assumption of raw consumption can be used as a worst-case scenario. Only two studies have tried to model the fraction of product eaten uncooked, unpeeled, and unwashed, in these cases by using a triangular distribution based on assumptions about the prevalence of such practices (Oron et al. 2010; van Ginneken and Oron 2000). Several studies do include a washing step before consumption (e.g. Navarro et al. 2009), but QMRAs that use specific data on the frequency or intensity of vegetable washing (e.g. in Barker et al. 2013; Domenech et al. 2013) are scarce as vegetable washing is not usually characterized by degrees but rather by yes (washed) or no (not washed) (e.g. Ottoson et al. 2011). However, in the study of Doménech et al. (2013) they used specific reduction data on the lettuce of the pathogen under study using varying dipping/rinsing times and different concentrations of sodium hypochlorite for disinfection, according to practices identified during a consumer behavior survey.

Table 6.5. Data needs related to consumer behavior and identified approaches (using genuine data, surrogate (S) data or assumptions (A)) to deal with these data needs.

Specific consumption data of the situation (country/region) under study	
(i) The use of relevant consumption data for the region/country and situation under study.	e.g. Hamilton et al. (2006b); Bastos et al. (2008); Ferrer et al. (2012)
(ii) The use of consumption data of another country as **surrogate** for the consumption patterns in the country relevant for the study (consumption size and frequency). E.g.:	
- The use of consumption data derived from the US DA or US EPA for another country/situation.	e.g. Barker et al. (2013); Oron et al. (2010); Mara and Sleigh (2010a); Navarro et al. (2009)
(iii) The use of **assumptions** on consumption portions and/or frequencies. E.g.:	e.g. Mota et al.(2009); Finley et al. (2009)
- 100 g of lettuce per person on alternate days or 150 days a year.	e.g. Shuval et al. (1997); Mara et al. (2007)
- Each consumption event (of lettuce) comprises 100 g.	e.g. Ottoson et al. (2011); Petterson et al. (2001); Petterson and Ashbolt (2001)
Information on consumer practices such as the prevalence, frequency, or intensity of vegetable washing and cooking habits in the population under study	
(i) The use of specific knowledge on household practices concerning the preparation of fresh produce in the community. E.g.: - Knowledge of washing practices in a community	e.g. Doménech et al. (2013); Barker et al. (2013)
- The use of specific consumption data of *raw* produce	e.g. Carrasco et al. (2010); Ferrer et al. (2012)
(ii) The use of **assumptions** or scenario analyses. E.g.:	
- Assuming that the vegetables under study are all consumed raw (e.g. lettuce, cucumber, but also broccoli or cabbage).	e.g. Hamilton et al. (2006a); Navarro et al. (2009); Al-Juaidi et al. (2010); Mota et al. (2009)
- Washing of produce by consumers was often included in scenario analysis (present or not), so true prevalence and efficiency was not accounted for.	e.g. Ottoson et al. (2011)

6.4.2.5. Selection of Dose-Response model in QMRA studies on water use in fresh produce

To calculate the risk of infection or illness, the selection of a dose-response model for use in QMRA is essential. Different dose-response models were used in the literature selected for review, including the exponential model (e.g. in Mota et al. 2009), the (simplified/approximated) β-Poisson model (e.g. in Mara et al. 2007), the β-binomial model (e.g. in Barker-Reid et al. 2010; Hamilton et al. 2006a) and the Weibull-Gamma model (Carrasco et al. 2010). Each model, of course, has its own inherent assumptions, e.g. on the distribution of the received dose and/or on the distribution of infection (Vose 2008). For some pathogens, different dose-response models were selected in different studies: e.g. for *Salmonella*, a Beta-Poisson dose-response model was selected by some studies (Stine et al. 2005b; Stine et al. 2011; Drechsel and Seidu 2011; Lim and Jiang 2013) and an exponential model was chosen by another (Puerta-Gomez et al. 2013). The preferred approach is the use of dose-response models that are based on information obtained during challenge (feeding) studies in human volunteers. However, for certain pathogens there are no feeding studies (usually due to ethical reasons), and in this case data can also be derived from epidemiological studies (or from animal experiments) (Kothary and Babu 2001). Nevertheless, a relevant dose-response model was simply not available for all pathogens under consideration (Table 6.6.).

An alternative approach is (again) the use of surrogate dose-response models. For example, in the case of QMRA studies for 'enteric viruses', this group of diverse viruses were treated as a single pathogen with a given dose-response model, e.g., rotavirus (e.g. Tanaka et al. 1998; Barker-Reid et al. 2010), used in QMRA. Since rotavirus was considered at the time to be the most infectious water and foodborne virus for which dose-response information was available, its use in this modeling was justified as providing a plausible upper-limit to the risk estimates (Haas et al. 1993). However with the recent availability of dose-response models for norovirus based on human challenge studies, norovirus may be a better 'reference' viral pathogen in the future (Teunis et al. 2008; Mara et al. 2010). Another example is the use of the dose-response model of *Shigella dysenteriae* and *Entamoeba coli* as surrogates, respectively, for *E. coli* O157:H7 (Ottoson et al. 2011; Danyluk and Schaffner 2011) and *Entamoeba histolytica* (Ferrer et al. 2012).

Table 6.6. Data needs identified to model the dose-response relation and identified approaches (using genuine data, surrogate (S) data or assumptions (A)) to deal with these data needs.

Dose-response data on the pathogen under study	
(i) The use of dose-response models for the specific pathogen under study, that are based on:	
- information obtained during human feeding studies	e.g. Barker et al. (2013); NRMMC-EPHC-AHMC (2006)
- data derived from epidemiological studies	
- animal experiments, if possible validated with outbreaks	e.g. Seidu et al. (2013) for *E. coli* O157:H7
(ii) The use of surrogate dose-response models. E.g.:	e.g. Tanaka et al. (1998); van Ginneken and Oron (2000); Petterson and Ashbolt (2001); Petterson et al. (2001); Hamilton et al. (2006a); Al-Juaidi et al. (2010); Barker-Reid et al. (2010); Munoz et al. (2010)
- To model the dose-response of 'enteric viruses', the group was treated as a single pathogen with a known dose-response model. Generally the dose-response model of rotaviruses is used, as rotaviruses have a low infectious dose and as such represent a worst-case situation.	
- The use of the dose-response model of *Shigella dysenteria* as a surrogate for the dose-response model of *E. coli* O157:H7.	e.g. Ottoson et al. (2011); Danyluk and Schaffner (2011)
- The use of the dose-response model of *Entamoeba coli* as a surrogate for the dose-response model of *Entamoeba histolytica*.	Ferrer et al. (2012)
(iii) When no dose-response studies or estimates/surrogates for the dose-response model are available, the use of a worst-case situation can be appropriate. This worst-case situation can be modelled by e.g. the use of the exact single-hit model with probability of infection =1 (r=1), which represents the maximum risk curve.	Seidu et al. (2008)

If no dose-response studies or estimates/surrogates for the dose-response relationship are available, a worst-case can be considered for modeling purposes. This was the case in the QMRA study done on *Ascaris* by Seidu et al. (2008). In this study, a worst-case situation was assumed by using the exact single-hit model with probability of infection equal to 1 (r=1) (Teunis and Havelaar 2000). A different approach was undertaken by Navarro et al. (2009), who developed a dose-response model for *Ascaris lumbricoides* concerning likelihood of infection in children (under 15 years old) from crops eaten raw (irrigated with wastewater). In this case, prevalence data obtained from stools of a large number of children in the Mezquital Valley in Mexico were used in conjunction with assumptions (e.g. on consumption) and surrogate data (e.g. to estimate amount of water remaining on produce and hence crop concentration). This dose-response model was also used in several other QMRAs (Mara and Sleigh 2010a; Navarro and Jimenez 2011; Seidu et al. 2013). An overview of these approaches is provided in Table 6.6.

In order to facilitate collection of data for dose-response modeling, most of the studies selected assumed that all strains of a certain pathogen are pathogenic/infectious to humans (e.g. Ottoson et al. 2011; Carrasco et al. 2010; Lim and Jiang 2013). This can be considered as a worst-case scenario as infectivity/pathogenicity for some microorganisms (e.g., *Salmonella* and *E. coli*) is indeed strain-specific (Ceuppens et al. 2011; Leimbach et al. 2013), and characteristic of the host (e.g., age, immune status, physical condition) (Kothary and Babu 2001). To take this into account, some studies have assumed 25% as a preliminary estimate for a reasonable range of the parameter values in infection probability for use in Monte Carlo simulation (e.g. Mara et al. 2007). For *Listeria monocytogenes*, a difference in susceptibility has been dealt with by using a different parameter values for high-risk and low-risk populations (Ding et al. 2013; Carrasco et al. 2010). The risk of cryptosporidiosis in immunocompromised people (such as HIV-infected individuals (Howard et al. 2006)) associated with park irrigation with reclaimed water was calculated assuming a minimum infective dose of 1/10 the dose for healthy individuals (Ayuso-Gabella et al. 2011). Other strategies for inclusion of the immunocompromised subpopulation in dose-response modeling have been used in studies outside the scope of this review (e.g. Howard et al. 2006; An et al. 2011).

A systematic assumption for dose-response models used in QMRA is that different exposure events are independent, hence there is no protective immunity in the target population (e.g. as mentioned by Ayuso-Gabella et al. 2011). This may be particularly important when using these models for estimating disease risk in developing countries, as the dose-response models for almost all pathogens are based on data collected from developed countries (Ferrer et al. 2012). Populations of developing countries tend to

experience higher exposure to many pathogens, and consequently high levels of immunity to certain pathogens may develop early in life (e.g. HAV and enteroviruses) (Hamilton et al. 2006b). Navarro et al. (2009) discussed the applicability of using their Beta-Poisson dose-response model for *A. lumbricoides* that was based on underlying data obtained from children in a population in which Ascariasis was endemic. They brought up that this model might not be directly applicable to a healthy population considering underlying immunity in the test population. However, attempts have been made in a study outside the scope of this review to include the effect of population immune status in dose-response modeling (An et al. 2011; Teunis et al. 2002a).

Another observation about dose-response models is that for most of the pathogens, dose-response data are available for a single isolate only. Volunteer studies with different *Cryptopsoridium parvum* isolates indicates that different isolates may produce different dose-response data and functions (Teunis et al. 2002b).

6.4.3. Expression of risk estimate and benchmarking to an acceptable level of protection

Amongst the forty one QMRA studies analyzed, some benchmarked their outcome to a defined objective or acceptable level of protection, while others provided the comparison of the annual probability of infection or illness using various scenarios or risk mitigation strategies as an outcome. The former appears to be more common in QMRA studies dealing with water treatment or water quality relative to QMRA studies dealing with food (Table 6.7.). In QMRA studies elaborated from the water perspective, one often refers to the benchmark level of acceptable risk once defined by the US EPA in its Water Standards (US EPA 1989). The US EPA considered at the time one infection per 10,000 individuals in a given year ($\leq 10^{-4}$ per person per year or abbreviated as $\leq 10^{-4}$ pppy) as a reasonable level of safety of drinking water. This number was derived in 1987 by determining the waterborne disease burden Americans already tolerated: the total number of reported cases of waterborne illness per year (then estimated to be 25,000) divided by the USA population (250,000,000 at the time) (Lechevallier and Buckley 2007). Another often cited acceptable risk level among the selected QMRA studies from a water microbiology perspective is limiting the maximum additional burden of water- and wastewater-related disease (provoked by use of reclaimed water) to 10^{-6} Disability Adjusted Life-Years (DALY) loss per person per year (pppy) (WHO 2004). The DALY metric has been introduced to enable comparison between the public health impact of various agents (e.g. microbial or chemical) and intervention options (Havelaar and Melse 2003). Both acceptable risk levels originate from WHO's water guidelines, and are integrated in QMRA studies on the risk of

consuming fresh produce irrigated with reclaimed water following the concept of the 'Stockholm Framework'. The 'Stockholm Framework' concept proposes that the tolerable health risks resulting from any water-related exposure (hence also irrigation water use in agriculture) should be the same (Fewtrell and Bartram 2001).

Not all QMRA studies from a water microbiology perspective based their risk estimates on an acceptable risk level (e.g. Asano et al. 1992; Finley et al. 2009). The formulae of annual risk of infection or illness, or the calculated loss of DALYs pppy can be used to evaluate and quantify the risks associated with the use of certain types of irrigation water or the consumption of certain types of vegetables. But these formulae can also be used in a different approach, e.g. the translation of a tolerable risk level to operational targets, such as targets for the irrigation water quality or for the efficiency of implemented risk mitigation strategies. This approach is more useful for establishing operational health-based targets (WHO 2006) and has been used in the report on Australian guidelines for water recycling (AGWR) (NRMMC-EPHC-AHMC 2006) and Stine et al. (2005b).

Despite the fact that several papers refer to the tolerable health risk set by the WHO of 10^{-6} DALY loss pppy, the DALY metric is generally not adopted. The reason for this is that the DALY metric requires additional information, such as the relationship between infection and illness, the disease burden, and the proportion of the population susceptible to developing disease following infection. A data gap identified in the selected literature is the absence of country-specific data for the calculation of disease burden (DALYs per case of illness of a certain pathogen). As such, epidemiological data needed for calculation of disease burden or values of disease burden itself are often obtained from other studies/countries and used as a surrogate for the situation under study (e.g. in Barker et al. 2013; Ayuso-Gabella et al. 2011; NRMMC-EPHC-AHMC 2006; Drechsel and Seidu 2011). This was also the case for those studies that calculated the tolerable annual illness or infection risk based on the WHO benchmark of acceptable risk (maximum additional burden of disease of 10^{-6} DALY loss pppy) (e.g. Lim and Jiang 2013; Mara and Sleigh 2010b). Finally, disease burden estimates have not been reported for all pathogens and/or they cannot be easily determined. This is the case, for example, for enteric viruses which cause diverse symptoms ranging from mild to severe (Hamilton et al. 2006a).

Table 6.7. QMRA studies with a Water-perspective that used one of the benchmark acceptable risk levels and their outcome.

RA studies with W-background	Outcome	Used benchmark acceptable risk level
Shuval et al. (1997); Tanaka et al. (1998); van Ginneken and Oron (2000); Hamilton et al. (2006a & 2006b); Diallo et al. (2008); Munoz et al. (2010); Navaro and Jimenez (2011); Ferrer et al. (2012)	Infection risk pppy	US EPA benchmark '$\leq 10^{-4}$ infection risk pppy'
Petterson et al. (2001a)	Likelihood of infection (number of people/ 10 000 exposed)	
Stine et al. (2005b); Stine et al. (2011)	Maximum concentration of pathogens allowable in water to meet acceptable risk level	
Seidu et al. (2013)	Number of days of irrigation cessation required to achieve annual tolerable infection risk	
NRMMC-EPHC-AHMC (2006)	Health based log reduction targets	WHO benchmark '$\leq 10^{-6}$ DALY loss pppy'
Ayuso-Gabella et al. (2011); Barker et al. (2013)	Annual burden of disease (DALY loss pppy)	
Mara et al. (2007); Bastos et al. (2008); Seidu et al. (2008); Al-Juaidi et al. (20101); Barker-Reid et al. (2010); Mara and Sleigh (2010b); Lim and Jiang (2013); Pavione et al. (2013)	Infection risk pppy	QMRAs that refer to the benchmark '$\leq 10^{-6}$ DALY loss pppy', but used as tolerable risk level a 'translated' tolerable infection risk pppy of this initial tolerable risk level of $\leq 10^{-6}$ DALY loss pppy.
Forslund et al. (2010); Forslund et al. (2012)	Disease risk pppy*	E.g. a tolerable infection risk of 10^{-3} pppy for rotaviruses and Cryptosporidium and 10^{-4} pppy for Campylobacter (WHO (2006)).

* used a maximum permissible annual diarrhoeal disease risk of 1×10^{-3} pppy, derived from the WHO benchmark 10^{-6} DALY pppy.

Table 6.8. Risk outcome of RA with solely a Food-background and their used 'acceptable risk reference'.

RA studies with solely F-background	Outcome	Used acceptable risk level
Mota et al. (2009)	Annual risk of infection from exposure to *Cryptosporidium or Giardia* through the consumption of tomatoes, or bell peppers, or cucumbers, or lettuce	at the end they mention that the U.S. EPA recommends that drinking water not pose an annual microbial risk of infection greater than 10^{-4}. But did NOT compare their risk estimates with this value.
Carrasco et al. (2010)	Mean number of cases of listeriosis per year in Spain due to ready-to-eat lettuce salads, and prevalence and concentration of the pathogen in the food at time of consumption.	A desirable general goal was a level of 100 CFU/g in the product at the time of consumption (as in regulation (CE) N° 2073/2005).
Rodriguez et al. (2011)	Estimates on concentration and prevalence of E. coli O157:H7 populations in commercially fresh-cut bagged lettuce (i.e. an exposure model).	/
Ottoson et al. (2011)	The probability of illness (P_{ill}) and number of illnesses per 10 000 servings.	Is absent, but is not relevant as the goal was to compare the relative difference due to different risk-mitigation strategies.
Danyluk and Schaffner (2011)	Number of illnesses	/
Ding et al. (2013)	Contamination level of lettuce at the time of consumption, probability of listeriosis illness per person per day eating lettuce, annual probability of listeriosis illness for consuming lettuce per person and annual cases of listeriosis per year in Korea	Compared contamination level of lettuce with food safety limit of *L. monocytogenes* on fresh produce fixed at 2 log CFU/g.
Domenech et al. (2013)	The mean, 5% and 95% percentile of probability of illness at home per person per serving depending on the initial load of lettuce at retail.	To comply with the U.S. Healthy People 2020 initiative which aimed to reduce the rates of listeriosis by 50 percent it was calculated that the probability of illness must be less than 1.32×10^{-8} listeriosis cases per serving to attain this level of protection.
Puerta-Gomez et al. (2013)	The probability of infection from a serving of ready-to-eat spinach.	Since more than 1% (i.e., 10^{-2}) of probability infection is considered unsafe for food processors, this value was used as the tolerance level in this study (=1.33 $\log_{10}$ CFU/g of sample)

Arguments for making the current tolerable risk levels less strict are available in literature (Haas 1996; Mara 2011). Haas (1996) has argued that some key factors used for the initial computation of the 1:10 000 level of acceptable risk may not be accurate. For example, computation of the currently used risk level from the late 1980s appears to have arisen partly because at that time, the perceived waterborne disease rate was 1 case per 10 000 people per year. But more recent assessments of the actual burden of waterborne illnesses appear to be much higher (Haas 1996; Colford et al. 2006). As such it may be that an annual risk of infection of 1 in 1000 (or even a less stringent risk level) is more appropriate than the current approach (Haas 1996). Mara (2011) advocates for lowering the benchmark of maximal additional burden of disease (a 10^{-6} DALY loss pppy) to 10^{-4} DALY loss pppy based on critical analysis of the basis from which the current benchmark is derived: i.e. US EPAs acceptance of a 70-year lifetime waterborne cancer risk of 10^{-5} per person (Mara 2011).

Several studies selected for this review objected to the use of stringent benchmarks of tolerable risk level. Lim and Jiang (2013) questioned the appropriateness of the US EPA $\leq 10^{-4}$ infection pppy risk benchmark in their efforts to assess sustainable water practices, such as the use of rooftop-harvested rainwater, for unrestricted irrigation of home-grown vegetables. In the context of wastewater irrigation, Mara et al. (2007) proposed that a less-stringent tolerable level for the risk of infection is of 10^{-2} pppy (i.e. once every 100 years, essentially once in a lifetime; or 1% of the community per year). This revised tolerable risk level was considered by several other studies (e.g. Barker-Reid et al. 2010; Pavione et al. 2013; Seidu et al. 2013; Seidu et al. 2008).

In food-oriented QMRA studies, benchmarking risk estimates to a tolerable risk target is uncommon, largely due to lack of an agreed upon set of food safety objectives or public health goals. In this review of QMRAs with solely a Food Science perspective, there was not even a standardized outcome expression for risk (Table 6.8.). For example, some studies calculated the number of illnesses to be expected from the consumption of a particular item among the population in a specific country/situation (e.g. Ottoson et al. 2011; Danyluk and Schaffner 2011); others calculated the probability of illness per serving (Domenech et al. 2013). The use of tolerable or acceptable risk values continues to be hotly debated in food safety circles, but it should be noted that “acceptability” is not only based on scientific data, but also on social, ethical and economic considerations, and thus is part of Risk Management and not of Risk Assessment (Reij and van Schothorst 2000). This is reflected in the majority of QMRA studies from a food perspective and reflects the purpose of such assessments, which is frequently about comparing potential risk mitigation strategies rather than coming up with specific regulations.

6.4.4. Risk Mitigation Strategies under consideration in selected QMRA studies and lessons learned

QMRA can be used as a tool to assess the impact of different risk mitigation strategies. Once the 'baseline' model is constructed, different scenarios can be evaluated and their relative impact on exposure or illness can be calculated (CAC 1999). The use of sensitivity analysis has also been acknowledged as an appropriate tool to identify possible risk management options (e.g. in Carrasco et al. 2010; Lim and Jiang 2013), while scenario analysis is used to compare mitigation strategies (e.g. in Rodriguez et al. 2011). But still, in decision making it is important to bear in mind that constructing a QMRA will always include a minimum number of assumptions which will contribute to the overall uncertainty, and decrease the reliability of conclusions drawn. When possible, validation of a model should be attempted. Interestingly, only one study explicitly stated that the model was validated using experimental values obtained in laboratory settings that was not included in the model; this was done using Standard Error of Prediction (SEP) method (Rodriguez et al. 2011). Some other studies compared the obtained level of infection/illness probability or number of illnesses with the actual situation presented by country-/region-specific disease statistics (e.g. in Carrasco et al. 2010; Ding et al. 2013). In this section, risk mitigation strategies that were investigated at the farm level (including selection criteria for irrigation water), at processing, and at the consumer level will be covered. Subsequently the overall lessons learned from the selected QMRA literature concerning the use of water in the fresh produce supply chain will be discussed.

At the farm level, different (waste)water treatment options were assessed to identify level of treatment necessary for irrigation of produce that is safe for human consumption (e.g. Munoz et al. 2010; Tanaka et al. 1998). Different types and contamination levels of (treated waste)water used for irrigation (e.g. Lim and Jiang 2013; Navarro and Jimenez 2011; Barker et al. 2013), and different national (e.g. Ottoson et al. 2011) and international (e.g. Bastos et al. 2008) criteria for irrigation water quality have been evaluated for their impact on food safety. But QMRA has also been used to evaluate the impact of **irrigation method** (e.g. drip, furrow or overhead) (e.g. Stine et al. 2005b); identify an appropriate withholding period (e.g. Stine et al. 2005b; Ottoson et al. 2011; Barker-Reid et al. 2010); investigate the possibility of crop selection/restriction (e.g. Bastos et al. 2008; Stine et al. 2005b); explore the use of biosolids having different microbiological contamination levels as soil amendments (Navarro and Jimenez 2011); and the value of microbiological criteria at primary production (Carrasco et al. 2010). In such studies, when the risk of different types of crops was compared, lettuce was frequently considered the most hazardous (e.g. Lim and Jiang 2013; Mota et al. 2009; Hamilton et al. 2006a).

At the processing level, the impact of washing and the use of disinfection treatments such as chlorination (Rodriguez et al. 2011) and ionizing radiation (Rodriguez et al. 2011; Puerta-Gomez et al. 2013) have been examined. Implementation of different sampling plans for batch acceptance by testing of final product (Rodriguez et al. 2011); reduction in maximum shelf-life indicated on package (Carrasco et al. 2010); a change in the packaging atmosphere (Carrasco et al. 2010); and the efficacy of cleaning and disinfection procedures (Rodriguez et al. 2011) on concentration and prevalence of pathogens or risk of illness have all been evaluated using QMRA. Carrasco et al. (2010) also modeled a hypothetical and ideal situation of 100% compliance with regulation (CE) N° 2073/2005, i.e. a concentration of *Listeria monocytogenes* in product at time of consumption of 100 CFU/g, as compared with the baseline model.

At the consumer level**,** the effect of produce washing and/or disinfection at home (e.g. Ottoson et al. 2011; Barker et al. 2013; Domenech et al. 2013); and risk communication strategies to reduce the probability of consumption of RTE lettuce salads by high-risk populations (relative to listeriosis risk) (Carrasco et al. 2010) have been assessed using QMRA. Sensitivity analysis suggested limiting serving size/consumption rate (Carrasco et al. 2010; Lim and Jiang 2013) and better home storage temperature control as potential mitigations for listeriosis (Carrasco et al. 2010). However the former was not further considered because of the broad health benefits of fresh produce consumption (Lim and Jiang 2013).

There are some key lessons learned from the selected QMRAs for each phase of the farm-to-fork continuum. At the farm level**,** *selection of an appropriate water source and/or degree of treatment* for irrigation water is critical. For instance, in a study by Ferrer et al. (2013), the use of canal surface water (wastewater) in Thailand for the irrigation of raw vegetables proved to result in a yearly infection risk of 100% for *Giardia* and *Entamoeba*. Also, the use of rooftop-harvested rainwater in the USA for overhead irrigation of home-grown lettuce, cucumber and tomatoes, led to unsatisfactory infection risks when compared to the US EPA risk benchmark (<1:10 000 pppy) for *Salmonella* spp. and *Giardia lamblia* (Lim and Jiang 2013). Barker et al. (2013) suggested three control points for domestic greywater reuse as irrigation water for home-produced fresh produce, being (i) appropriate choice of greywater source (bathroom water preferred above laundry water); (ii) "opting out" of greywater use on days when a household member is ill; (iii) the use of biocides (particularly in laundry water) could reduce microbiological contamination of greywater.

Use of non-disinfected secondary treated reclaimed water for fresh produce irrigation leads to an unacceptable high annual risk of infections (Tanaka et al. 1998; Hamilton et al.

2006a). Increasing the microbial removal efficiency of wastewater treatment is associated with a reduction in public health risk associated with fresh produce consumption (e.g. in Al-Juaidi et al. 2010; Pavione et al. 2013). Therefore, the use of a tertiary treatment step with disinfection was judged necessary to adequately manage infection risks of e.g. enteroviruses when eating fruits and vegetables (Munoz et al. 2010).

In several QMRA studies, the objective was to set the health-based log reduction target necessary to meet a predetermined tolerable risk (e.g. in NRMMC-EPHC-AHMC 2006). In an Australian context, viruses required the highest (6.1) $\log_{10}$ reduction to meet the health target. This due to the high concentration of viruses in domestic wastewater, but also due to their low infectious dose compared to bacteria (reduction of 5.0 $\log_{10}$ *Campylobacter* was needed) and the high disease burden compared to protozoa (reduction of 4.8 $\log_{10}$ *Cryptosporidium* was needed) (NRMMC-EPHC-AHMC 2006). These findings are consistent with the higher disease risk for viruses relative to other enteric pathogens generally obtained when QMRA was performed for different classes of pathogens (e.g. in Mara et al. 2007; Pavione et al. 2013; Bastos et al. 2008). However, this 6 $\log_{10}$ reduction required for e.g. enteric viruses does not solely have to be obtained using water treatment options, but other non-treatment options (e.g. different irrigation practices, implementation of a withholding period, post-harvest processing) can also be part of a multi-barrier approach (Drechsel and Seidu 2011). Stine et al. (2005b) used QMRA to help set irrigation water quality standards for enteric bacteria and viruses. When furrow irrigation was used for production of cantaloupe or lettuce (and a worst-case scenario in which produce is harvested and consumed the day after the last irrigation event and maximum exposure is assumed), 2.5 CFU/100 ml of *Salmonella* and 2.5 x 10^{-5} most probable number per 100 ml of hepatitis A virus would be the maximum concentration allowable in irrigation water to ensure an annual risk of ≤1:10 000 (Stine et al. 2005b).

The second important factor at the **farm level** is the use of appropriate *water application practices*. Firstly, the selection of an appropriate *type of irrigation method* must be considered. Several QMRA studies modeled the impact of different irrigation practices (e.g. Stine et al. 2005b; Van Ginneken and Oron 2000; Al-Juaidi et al. 2010). Only a minority were actually based on experimental studies (e.g. Stine et al. 2005b). The study by Stine et al. (2005b) suggested that subsurface drip irrigation reduces the risk of crop contamination compared with furrow irrigation. However, the impact of irrigation method on microbial contamination can be crop-specific. For instance, contamination of fresh produce by contact with irrigation water can be dependent on the physical properties of the edible portion of the plant, such as surface texture, and the location of the edible portion of the plant in reference to the irrigation water (Stine et al. 2005b). High and low growing

crops represent different soil-effluent-plant contact situations (Bastos et al. 2008; Pavione et al. 2013). For example, in contrast to cantaloupe and lettuce, no microorganisms (PRD1 phage or *E. coli*) could be detected on bell peppers after subsurface drip or furrow irrigation, which is a typical example of a crop growing relatively high above ground (Stine et al. 2005b). Comparatively speaking, lettuce has the highest infection risk (e.g. in Hamilton et al. 2006a; Bastos et al. 2008; Mota et al. 2009; Lim and Jiang 2013) due to its relatively high water retention rate. Consequently, crops selection has been identified as an effective complementary health hazard control measure (e.g. Bastos et al. 2008).

Another aspect of water application practice is the selection of a *withholding period*. Generally a withholding period has a positive influence on risk and has been identified as a risk mitigation strategy (e.g. in van Ginneken and Oron 2000; Hamilton et al. 2006a). However, the magnitude of the influence of the withholding period is dependent on the environmental conditions, the quality of the water used, and the pathogen of interest (Hamilton et al. 2006a), as some pathogens have a relatively long survival time in the environment (e.g. *Ascaris lumbricoides* and protozoa) compared to others (e.g. *Campylobacter*).

In addition to irrigation, water can also be used for *delivery of e.g. pesticides* in the form of a spray whereby the spray can make direct contact with the edible portion of the plant, serving as a source of pathogen contamination. Hence, pathogen concentrations in water used for e.g. pesticide dilution should also be contained in order to comply with acceptable risk levels (Stine et al. 2011).

At the **processing level,** water can be part of a risk reduction strategy, but when no appropriate sanitizer is used, can contribute to cross-contamination (discussed in Chapter 5). Several studies included washing for risk reduction during processing (e.g. Carrasco et al. 2010; Puerta-Gomez et al. 2013). However, commonly used packinghouse practices (water washing, and liquid sanitization treatments using chlorine) are not adequate to ensure the safety of the produce when initial or cross-contaminated microbial loads are elevated (Puerta-Gomez et al. 2013). Generally, washing results in a microbial reduction of ca. 1-2 $\log_{10}$ (Lopez-Galvez et al. 2009), remembering that the washing step has also been identified as a possible route of cross-contamination. The model of Danyluk and Schaffner (2011) predicts that a majority of simulated cases of illnesses in the 2006 *E. coli* O157:H7 spinach outbreak arose from leafy greens cross-contaminated during washing. In a study by Rodriguez et al. (2011), a noticeable reduction in the number of lettuce bags contaminated with *E. coli* O157:H7 resulting from cross-contamination was obtained when preserving a concentration of 50-200 ppm of chlorine in the washing bath, when compared to the baseline scenario which did not include any intervention step. However, if the initial

batch entering the production line was highly contaminated (e.g. 100 cfu/g), chlorination (200 ppm) was not as effective in reducing cross-contamination compared to the scenario of a lower contaminated initial batch. Even when all possible interventions were performed (e.g. washing with chlorine, irradiation, sampling plans), there remained a small probability of *E. coli* O157:H7 contamination, confirming that zero risk does not exist.

Traditionally, microbiological criteria have been established to improve food safety. However, it has been suggested that these are less effective at managing risk when low levels of contamination are considered, which is the most likely situation under most field and processing conditions (Rodriguez et al. 2011). Carrasco et al. (2010) suggested that other measures such as the use of a modified atmosphere packaging, or reduction of the product's shelf-life may be more effective in reducing the number of listeriosis cases, for example, associated with consumption of ready-to-eat lettuce salads in Spain.

Finally, at the consumer level, several QMRA studies have identified produce washing as an important intervention step for lowering enteric disease risks associated with the consumption of fresh produce (e.g. Navarro et al. 2009; Ayuso-Gabella et al. 2011; Domenech et al. 2013). For example, in the QMRA model of Ottoson et al. (2011), rinsing for 15 s under running tap water gave rise to an average six-fold reduction in the risk of illness associated with *E. coli* O157 contaminated lettuce. In an Australian study, washing of lettuce was estimated to reduce the burden of NoV illness due to home-produce lettuce consumption (irrigated with greywater) by ca. 1.5-2 $\log_{10}$ DALYs pppy (Barker et al. 2013).

6.4.5. Further perspectives in risk assessment related to use water and safety of fresh produce

From a public health point of view, the disease syndrome (e.g., gastroenteritis or diarrheal disease) may be of greater interest than a specific cause of disease (e.g., noroviruses). In this case, a multi-risk assessment approach is sometimes undertaken. For example, Diallo et al. (2008) developed a risk assessment that included *Cryptosporidium*, *Giardia* and diarrhegenic *Escherichia coli* to assess the infection risk of diarrhea-related pathogens in a tropical canal network. The choice of pathogens was justified as these were estimated to be the etiological agents responsible for ~47% of diarrhea in Thailand. Unfortunately the risk for infection with these pathogens by consumption of 100 g of irrigated vegetables was assessed individually and not combined. In literature outside the scope of this review (e.g. de Man et al. 2014), there are examples in which health risk due to ingestion of urban floodwater was assessed by determining the risk of infection for a set of waterborne pathogens that can cause gastrointestinal diseases. The overall risk of infection per

exposure event was calculated and comprised the risk of infection with *Campylobacter jejuni* and/or *Cryptosporidium* and/or *Giardia* spp., and/or noroviruses, and/or enteroviruses. The use of the DALYs approach for these purposes has also been proposed, as this possesses the flexibility to aggregate all the risks presented by different pathogens to one single DALYs value (Lim and Jiang 2013).

To assess the risk on human health due to consumption of e.g. vegetables irrigated with (treated) wastewater, ideally both chemical and microbiological risks should be assessed simultaneously and perhaps cumulatively. In a study by Munoz et al. (2010), both chemical and microbiological (enteroviruses) risks are evaluated in parallel. The possibility of analyzing these risks from a cumulative point of view using the concept of disability adjusted life years (DALYs) was stated, but it was concluded that this was not possible due to lack of available DALY values for many microorganisms as well as for the organic pollutants included in the study.

When evaluating (treated) wastewater as a water source for irrigation and hence contamination, intake of more than one produce type would be relevant. This approach can be referred as multi-source exposure calculations. An example of this approach is provided by Pavione et al. (2013). These investigators calculated the risk of infection for each of the reference pathogens (rotavirus, *Campylobacter*, *Cryptosporidium*) by consuming low-growing salad crops and high-growing crops. Each of these groups consisted of various kinds of vegetables of which mean consumption data per person per day was available.

Exposure to pathogens (e.g. present in wastewater) can occur via multiple pathways. When risk of infection by multiple pathways is estimated as a single risk outcome, the process can be defined as multi-pathway risk assessment. An example can be found in a QMRA study done by Seidu et al. (2008). These investigators examined the risk of exposure associated with consumption of wastewater-irrigated lettuce, but also the risk from exposure of farmers' due to both accidental ingestion of irrigation water and contaminated soil. The latter resulted in a combined annual risk of infection from exposure to wastewater and contaminated soil for the farmers. Note that these multi-hazard, multi-pathway and multi-source QMRAs are a relatively new approach and will need further development to expand their usefulness.

6.5. CONCLUSIONS

The use of QMRA to manage fresh produce safety risk is complicated by a vast number of produce items, production/processing conditions, as well as the lack of supporting data. Still, the selected QMRA studies discussed here demonstrate its use in specific situations, in some instances in support of decision-making on the use, quality and treatment of water used across the fresh produce supply chain. Overall having analyzed the selected QMRA studies it can be concluded that viruses often resulted in the highest risk estimates and leafy greens were the commodity of greatest concern. With regard to other aspects on the use of water in the fresh produce chain, cultural differences in food preparation, the susceptibility of different populations and regional variation in the prevalence and concentration of pathogens in (waste) water and environmental conditions means that the results obtained by one QMRA study cannot always be translated to other situations or regions.

There are many sources of uncertainty that might arise from inputs to a risk assessment, e.g., measurement errors, sampling errors, systematic errors, estimated (using surrogates) or excluded variables, incorrect model forms and abnormal conditions (Haas et al. 1999). The QMRA models are constructed based on the best knowledge and available information (parameters and data) at the time of development. For example QMRAs that make use of ratios to estimate the concentration of pathogens in water may need to be revised if better data become available. Especially for uncultivable viruses such as NoV the performance of a well-funded QMRA is challenging. Since not only is there an apparent lack of relevant data on prevalence and concentration of the relevant enteric virus in the environment or food, but also data interpretation is complicated since the detected genomic copies are not necessarily infectious. As detected RNA could originate from defective virus particles. Also the frequent use of surrogate data concerning inactivation and persistence, and the use of assumptions mount up to a high level of uncertainty when enteric viruses are the pathogen under study. It should also be noted that only two of the 25 studies that included viral pathogens focused on NoV, while NoV are identified in this PhD study as the most relevant viral pathogen concerning fresh produce. Hence a change in focus and collection of more relevant data is needed.

QMRA studies are particularly useful in evaluating different control scenarios, but as the outcomes rely partly on assumptions, results should be interpreted as an indication of the level or degree of safety and not as absolute values. Still, the outcomes of these exercises can be used to guide the risk management in preventing contamination, controlling it if it occurs, and identifying areas in need of further research or data collection. Overall, the use of QMRA is leading to more flexibility and more tailored guidelines on water treatment

and levels of pathogens in irrigation or processing water for the fresh produce in certain regions. Drawbacks are however, capacity and knowledge to perform the QMRA and the need for data relevant the specific regions.

CHAPTER 7

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

7. GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

7.1. RELEVANCE OF NOROVIRUSES IN FRESH PRODUCE

NoV are responsible for 47-96% of all reported acute gastroenteritis outbreaks in countries all around the world (Atmar and Estes 2006). However the majority of NoV outbreaks is due to person-to-person transmission, mostly situated in health care institutions. Only a fraction, approximately 22% of NoV outbreaks, is due to foodborne transmission (Hall et al. 2014; Verhoef et al. 2009). The most common contributing factor to NoV food contamination is the infected food handler (EFSA and ECDC 2014; Hall et al. 2014). As such, in the USA infectious food workers were implicated as the source of contamination in 70% of NoV foodborne outbreaks (FBO) that reported contributing factors (Hall et al. 2014). The importance of the food handler is also demonstrated in statistics that present the most popular settings and the most identified food vehicle in NoV FBO. As such, in the USA 64% and 17% of NoV FBO (2009-2011) found place in restaurants and catering or banquet facilities, respectively (Hall et al. 2014), and the most frequently identified food vehicles in NoV FBO in e.g. the EU (2012) were mixed food (33.0%) and buffet meals (20.6%) (EFSA and ECDC 2014). However, still an important part of NoV FBO can be linked to specific food commodities for which contamination can occur further down the food chain, up to the point of origin. The latter is especially relevant for e.g. shellfish grown in fecal contaminated waters and frozen berries contaminated by infected pickers. NoV FBO can also be divided in outbreaks due to Food of Animal origin (FoAO), that are mostly linked to contaminated shellfish, and outbreaks due to Food of Non-Animal Origin (FoNAO). In Europe (2007-2011), NoV outbreaks due to FoNAO (n=75) were mostly attributed to contaminated fresh or frozen soft berries (29/75, 39%) and leafy greens eaten raw as salads (24/75, 32%). In general outbreak statistics for FoNAO in Europe (2007-2011), NoV were identified as the top cause of outbreaks (n=75), responsible for 34% (75/219) of all outbreaks due to FoNAO (EFSA 2013).
In the present PhD project the focus was put on the food safety issue of NoV in foods of non-animal origin, in particular berries and leafy greens.

Despite the well-deserved notorious reputation of NoV among FBO specialists, public awareness of this viral threat, and overall food safety issues with microbiological hazards in fresh produce, was almost non-existent at the start of this PhD project in August 2010. However, the latter has changed during the course of this PhD thesis as the European public was informed on the implication of fresh produce as a potential source of foodborne pathogens during the German EHEC crisis in 2011 (Buchholz et al. 2011). In particular for

NoV this was reinforced by a massive NoV outbreak affecting over 10 000 schoolchildren in 2012 due to contaminated strawberries as well as by rapid alerts and media attention due to the recent outbreak due to HAV in soft red fruits all over Europe (2013-2014) (EFSA 2014; Mäde et al. 2013).

Increasing linkage of NoV and fresh produce such as soft red fruits and leafy greens has prompted an increase in analyses performed in several countries, giving a boost in the number of RASFF alerts on NoV. Furthermore **import requirements** concerning strawberries from China, have been imposed since January 2013 following the large NoV outbreak in Germany 2012, enforcing to sample 5% of consignments of (frozen) strawberries imported from China into the EU to be tested for the absence of NoV and HAV in 25 g (n=5, c=0) (Anonymous 2012a). These import requirements represent the first regulation to specifically incorporate the sampling and testing for NoV and HAV in foods and hence stating that these pathogens should be absent in food.
On a European level the interest in soft fruits and leafy greens as vehicle for FBO due to NoV is also demonstrated by the recent publication of two **scientific opinions** by EFSA, the European Food Safety Authority, on *Salmonella* and Norovirus in berries (EFSA BIOHAZ Panel 2014a) and *Salmonella* and Norovirus in leafy greens (EFSA BIOHAZ Panel 2014b).

The increasing recognition of the relevance of NoV in food and fresh produce is also noticeable in the number of international and national research project devoted to the topic of NoV or that included NoV as a relevant foodborne pathogen. As such, capacity building and data gathering concerning NoV in fresh produce was the goal in several **European projects**, e.g.: COST 929 – ENVIRONET (2006-2010), VITAL (2008-2011, FP7, Grant no. 213178), BASELINE (2009-2014, FP7, Grant no. 222738). The present PhD thesis was possible because of an IWT personal scholarship grant but also due to partial funding from the European project ***Veg-i-Trade*** (2010-2014, FP7, Grant no. 244994) and the SPF Public Health project TRAVIFOOD. Also in the USA large budgets are reserved for NoV research, as the USDA's largest grant ever for food safety, comprising $25 million, has been assigned to the project NoroCORE (2011-2016), a research project on Norovirus Collaborative for Outreach, Research, and Education (website: http://norocore.ncsu.edu/). Extensive research has resulted in an exponential increase in published data concerning detection, persistence, inactivation, removal and presence of NoV in fresh produce. In **Chapter 1** of this PhD thesis an effort was made to give an extensive overview of the literature concerning the prevalence and transmission of NoV in the farm-to-fork chain of fresh produce and the relevance of NoV as foodborne pathogen in fresh produce.

In conclusion, the FBO events of the last years and resulting media attention in combination with an increasing body of literature on the prevalence, concentration and transmission of NoV in the fresh produce chain, ensure that the topic of this PhD thesis can be considered highly relevant and timely.

7.2. CHALLENGES AND UNCERTAINTIES RELATED TO NOROVIRUS DETECTION, INTERPRETATION AND CONTROL

When striving for a holistic approach to study and to understand NoV in a farm-to-fork chain, one is confronted with several uncertainties, as was the case during this PhD study concerning the two case studies lettuce and raspberries. These uncertainties and hence challenges are schematically depicted in Figure 7.1. and will be discussed in this paragraph.

Note that in this PhD study 'NoV detection' implies the detection of human infecting NoV of GI and GII. Human NoV of GIV were not considered during method evaluation (Chapter 2 & 3) and screening (Chapter 4) in this PhD study, since the most relevant human NoV causing FBO are considered to be GI and GII human NoV. Inclusion of GIV human NoV in method evaluation would have been very challenging as fecal samples of this rare genogroup would have been needed for inoculation and in case of screening, detection of GIV human NoV would have requested an additional real-time PCR reaction step with specific primers and probes. Given that GIV has to the best of my knowledge not yet been linked to a FBO, inclusion of GIV human NoV was not considered relevant for this PhD study. Moreover also the current ISO/TS 15216-1 method for detection of NoV in food solely focuses on detection of GI and GII human NoV.

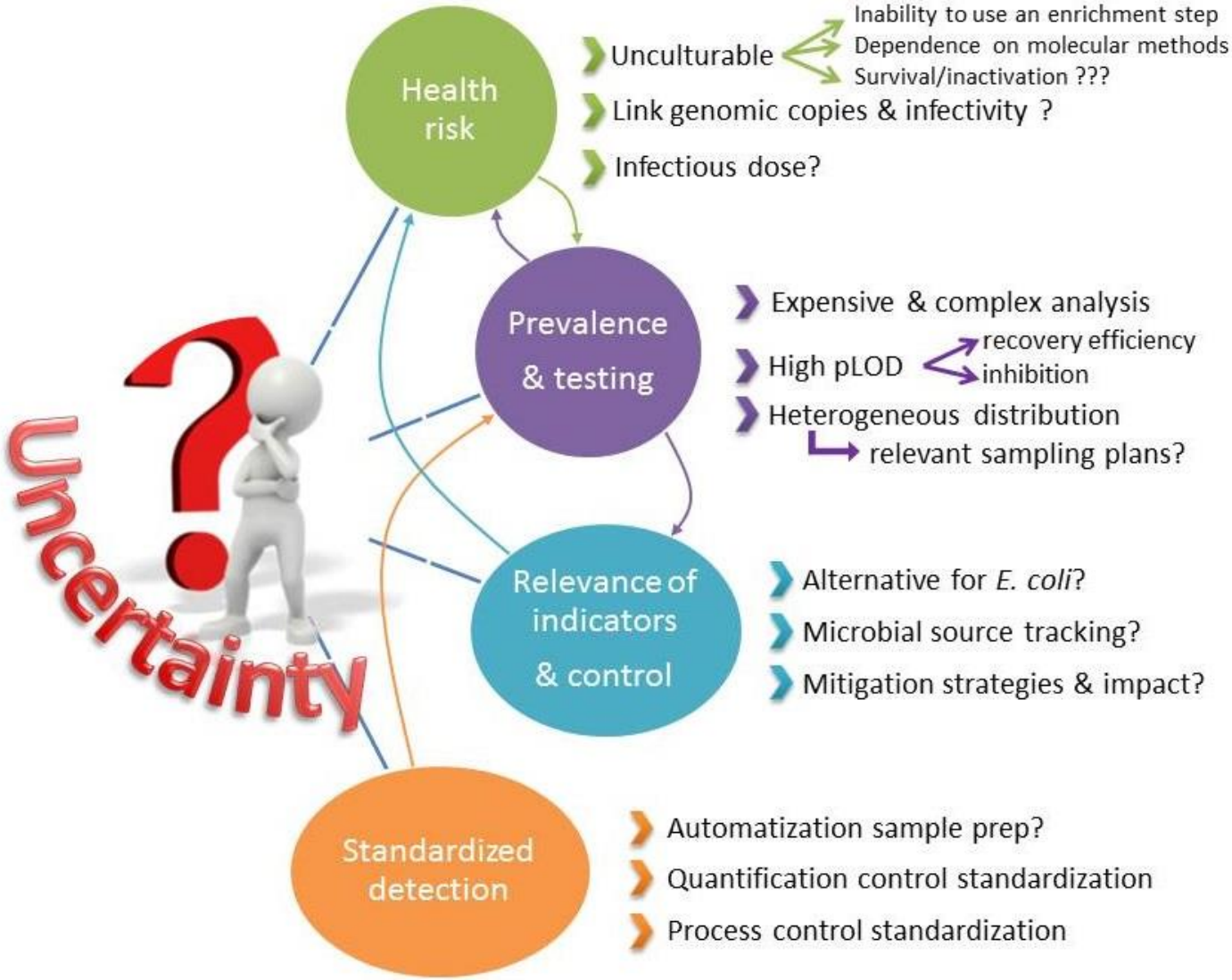


Figure 7.1. Mapping the uncertainties and linked challenges concerning the study of NoV in a farm-to-fork chain. These uncertainties are interconnected as they have impact on each other. Examples of such relations are represented by arrows. pLOD: practical limit of detection; *E. coli*: *Escherichia coli*.

HEALTH RISK

In contrast to classic bacteriology, detection of enteric viruses such as NoV is based on the detection of nucleic acids (RNA) due to the unavailability of an appropriate cell culture assay for NoV detection. Hence **molecular methods** are needed among which RT-qPCR is considered to be the gold standard. However the major drawback related to molecular methods is the **inability to differentiate** between RNA detected from **infectious viral particles, defective viruses** and free RNA. This is an important issue as it has been unequivocally demonstrated that the RNA associated with inactivated viruses remains detectable by nucleic acid amplification long after viral infectivity has been lost (Baert et al. 2008d; Knight et al. 2013). Therefore the 'history' of the analyzed samples, e.g. performed treatments such as High Pressure Processing (HPP), heat treatment and irradiation, should be taken into account during analysis of the results obtained by RT-qPCR. Hence interpretation of results based solely on the presence of nucleic acids does imply some uncertainty concerning the related health risk. This further complicates legal implementation and enforcement of microbiological criteria concerning foodborne viral pathogens such as NoV and HAV.

As a solution for lack of a cell-culture, prediction of NoV infectivity has been attempted using assays to measure the integrities and/or functions of viral RNA molecules and capsid proteins, which are the two essential parts for an intact and infectious virus particle (Knight et al. 2013). As such, in literature several inactivation studies are available for human NoV using these assays, e.g. use of porcine gastric mucin-conjugated magnetic beads (PGM-MBs) by Dancho et al. (2012), and long-range and binding RT-qPCR assay have been used in our lab at Ghent University on positive raspberry samples identified in the batch screening in Chapter 4 (reported by Li et al. 2014a). Using these assays, Li et al. (2014a) demonstrated the abundant presence of NoV particles with intact receptor-binding sites and a large part of complete genome in the positive NoV samples of Company A (i.e. mixed/minced raspberries intended for production of puree). However, these alternative assays are not yet satisfactory and should be validated with human infectivity studies for each inactivation method as different inactivation methods can have different effect on nucleic acids and/or capsid proteins and hence not each assay is appropriate for detecting all types of inactivation mechanisms. For example when using a PGM-MB binding assay this assay exploits the loss of virus binding ability to porcine gastric mucin as means of eliminating virions that have been rendered noninfectious by capsid damage from subsequent RT-PCR assay. However, next to the inability to assess damage to nucleic acids it is highly possible that not all forms of protein or capsid damage possibly leading to

virus inactivation will prevent porcine gastric mucin (PGM) binding and hence will be detected by this assay (Kingsley et al. 2014).

As such, due to remaining uncertainties and the fact that conducting these assays could result in a higher LOD and is even more laborious and time consuming, these assays have not yet been used in screening situations. Hence **continuous effort should be done to find an appropriate and validated cell culture assay for NoV** to tackle this major uncertainty concerning health risk.

STANDARDIZED DETECTION

Since the boom in molecular methods in the 90s the detection of 'non-culturable' NoV in small quantities as present in food became possible. However research on NoV extraction and concentration has of old focused primarily on water matrices and on shellfish and hence the development of extraction methods for matrices such as ready-to-eat food and fresh produce only started 10 to 15 years ago. As such **current extraction methods are relatively new.** To put into perspective, in Belgium detection methods for foodborne NoV were only implemented in national reference laboratories since 2006, coinciding with the first PhD project on NoV detection in food of our Ghent University Department of Food Safety & Food Quality by dr. Leen Baert (Baert 2009). However since the implementation of the detection protocol NoV has become one of the most frequently detected foodborne pathogens in verified FBO in Belgium i.e. associated with 7/27, 2/28, and 9/32 of characterized outbreaks reported in 2010, 2011, and 2012, respectively (FAVV 2014).

As a result of efforts made within various EU projects, between reference laboratories and within the European Committee for Standardization/Technical Committee/working Group 6/Task Group 4 on virus detection in food (CEN/TC275/WG6/TAG4), in July 2013 an ISO Technical Specification (TS) was launched for the determination of HAV and NoV in soft fruit and salad vegetables, bottled water, bivalve molluscan shellfish, and on food surfaces, using real-time RT-PCR (RT-qPCR) (ISO/TS 15216-1:2012) (Anonymous 2012c). This is, however, still a technical specification, meaning that further improvements to the proposed protocols can still be inserted, and as such further research into these detection protocols can contribute to a better proposal, and over time, an optimized international standard. The availability of an ISO protocol, even though the methods are not perfect, is anyway a significant step forward as it allows to assess newly proposed methods in a more standardized way by comparing the new proposal to the ISO (e.g. in Coudray et al. 2013). The provision of an ISO/CEN reference method also provides an overall accepted method

for competent authorities and companies and service laboratories to use. It facilitates an equal playing ground for NoV testing in (international) trade and enhances harmonized collection of NoV data in monitoring and surveillance programs with inclusion of the necessary controls.

At the start of this PhD project, the literature was explored for methods qualified for the examination of the whole farm-to-fork chain of fresh produce with relevance to the presence of foodborne viruses. An in-house detection method for viral detection in fresh produce, developed and tested during previous PhD research at our lab in Ghent University (Baert 2009; Stals 2011), was already available and in agreement with the detection strategy proposed in the CEN protocol. Hence this in-house detection method was used for detection of NoV in the two case studies of this PhD thesis, i.e. raspberries and lettuce. Despite the identification of irrigation water as a transmission route for NoV contamination of fresh produce the former CEN protocol and the current ISO/TS 15216-1 protocol consistently lack in description of methods for the **detection of foodborne viruses such as NoV in irrigation waters and process water**. Although there is a need for detection methods for viruses in non-potable waters such as surface water, collected rainfall water (to be used as irrigation water) or food processing water for pro-active testing in the frame of assessing the use of clean water in the framework of good agricultural or good manufacturing practices. Detection methods in (surface)water would also enable to collect data up front in the food supply chain to use for risk assessment studies or to enable source tracking in the frame of a preventive approach. The ISO/TS 15216-1 protocol primarily focuses on food and hence only a viral concentration method for bottled water is included. Also in literature it was apparent that the primarily focus of studies and protocols for the detection of viruses in water at that time was rather on source water for drinking water production, wastewaters and recreational waters but not that much on water sources used in the fresh produce supply chain. Hence in **Chapter 2** of this PhD study the performance of four methods for detection of foodborne viruses and surrogates were compared in four different irrigation water types (2-5 Liter samples) and in post-harvest washing water used in the fresh-cut produce industry (1 Liter samples). The selected viral concentration method consisted of an electronegative membrane in combination with an alkaline elution buffer based on Triton X-100 (a surfactant) and a secondary concentration method based on precipitation with PEG. This method termed ‘Method 1’, based on Hamza et al. (2009) and Katayama et al. (2002), obtained mean sample recovery efficiencies for MNV-1 between 4.8% - 21.9% depending on the type of water matrix, and obtained similar recovery efficiencies for GI and GII NoV.

Also in case of FBO investigations (Boxman et al. 2011) and in the framework of a preventive approach it can be useful to do environmental testing. In **Chapter 3** an **improvement** is proposed to the currently applied ISO/TS 15216-1 for the **analysis of surfaces using swabs as implement**. Both the selected method for detection in water and the improved semi-direct swab lysis protocol were applied in the Veg-i-Trade project.

During this PhD research, experience was acquired in performing viral extraction methods from different matrices and some concerns on the standardization of currently proposed viral extraction methods (ISO/TS 15216-1) were identified. As such, the sample recovery efficiency (SRE) of a method is influenced by several factors including the efficiency of the virus elution from the berries, the virus concentration, and the RNA extraction. However also the food/water matrix, the virus type (Dubois et al. 2002) and viral suspension used can have an effect on the SRE. The latter was suggested in **Chapter 4** as a possible reason for the difference in SRE obtained with two seemingly similar batches of PC virus. This has also been suggested in literature for detection in water (Gassilloud et al. 2003; Haramoto et al. 2007) and food samples (Sanchez et al. 2012), since a virus suspension may contain free RNA molecules and defective particles that are detected in the positive control if submitted to RNA extraction alone. Whereas in food and water samples those free RNAs and possible also the defective particles are most likely lost during the concentration step (Sanchez et al. 2012). Hence differences in virus particles to genomic copies ratio in the viral suspension used as PC can lead to heterogeneity of obtained SRE even though a similar detection strategy was implemented. As such in Chapter 4 a proposal for more research on the impact of PC batch quality is requested to further verify this hypothesis and to enable the independent production of **reliable quality control materials** needed for better benchmarking of the resulting SRE between analyses. Also the availability of reliable **nucleic acid quantification standards**, which require a minimum of handling to minimize variations, is challenging. The availability of reliable quality control materials, has also been identified in the recent EFSA opinion on NoV in leafy greens as a necessary prerequisite before there can be complete confidence in the concordance of results within and between laboratories (EFSA BIOHAZ Panel 2014b).
However, despite the availability of standard material for quantification in a recent EU reference laboratory proficiency test (PT 46) for detection of NoV and HAV in shellfish, quantification of a LENTICULE^{TM} disc still resulted in a highly variable outcome which ranged up to 3 $\log_{10}$ units difference among participating labs (Cefas 2013). This indicates that exact and reliable quantification is still a challenge, even in reference laboratories. Next to **variability in quantification**, variability in obtained SRE within and between labs is also observed. The **variability in SRE** obtained during different experiments/studies at

this lab is illustrated in Table 7.1. Also in literature high standard deviations are observed during sample analysis (e.g. SD of 32.7% for detection PC (bovine enterovirus type 1) in lettuce and SD of 20.9% for detection PC in red fruits in Loutreul et al. (2014)). Hence further standardization of both sample preparation as well as the detection step and the necessary controls is required to further improve the accuracy of NoV quantification in food and environmental samples and hence improving concordance of results between laboratories. However note that greater harmonization would not necessarily result from application of the ISO technical specification alone as this ISO/TS allows the use of e.g. different kinds of RNA extraction methods/reagents and different kinds of qPCR detection reagents and possibilities (1-step/2-step). Remark also that international inter-laboratory validation of the ISO/TS 15216-1 method is still pending (Cefas 2014). Hence the range of possible variation in outcome when different labs interpret and implement this ISO method in order to detect NoV is not fully investigated and understood. This information is however needed, together with information on the practical limit of detection and limit of quantification of these methods that should be demonstrated by a collaborative trial or validation.

Public awareness and increasing linkage between viral pathogens and certain fresh produce commodities such as soft red fruits, has put pressure on the availability of the detection methods. As such a method that was until recently only applied in research, is now widely requested in relation to import controls and commercial supplier agreements. With an ISO/TS only just in circulation it is important that sample analysis facilitating labs possess the right skill set, experience, and have properly validated the detection strategy for internal use of the standard in their specific lab. To assure accuracy, the detection strategy demands the inclusion of several controls such as a process control to control the sample extraction efficiency, amplification control, and controls to control for good practices (i.e. no contamination). However extra care should be considered in relation to false positive results due to **cross-contamination** since molecular methods are particular prone to contamination. The detection of false positive IQF raspberry samples in **Chapter 4** upon sequencing of the amplicons indicated that even in situations of adherence to good laboratory practices, one must be vigilant of the possibility for cross-contamination since contamination can occur randomly and hence cannot be excluded or necessarily detected by the use of negative controls. Also other labs experience cross-contamination since in the EURL proficiency testing (PT 46) the false positive reporting rates for GI and GII NoV were 5% and 7% respectively for LENTICULES (Cefas 2013).

Table 7.1. Sample recovery efficiency for detection of MNV-1 as process control (PC) or reverse transcriptase control (RTC) obtained in this lab.

	Description of analyzed samples	Dilution	PC recovery	RTC recovery	Reference
Water (Figure 7.2)	Open well water (5L, Method 1) (n=3)	1/667	4.8% ± 2.9%	/	Chapter 2 (in 2011)
	Open well water (2-5L, Method 1) (n=27)	1/667	1.5% ± 1.5%	/	Veg-i-Trade (in 2012)
		1/6667	3.0% ± 3.1%	/	
Raspberries (Figure 7.3)	Raspberry puree (10g) Poland (n=70)	1/500	4.7% ± 4.2%	40.2% ± 16.6%	Chapter 4 (in 2013)
	Raspberry IQF (10g) Poland → n=45 (9 batches)	1/500	2.4% ± 2.6%	75.7% ± 20.0%	Chapter 4 (in 2013)
	→ n=15 (3 batches)	1/500	28.7% ± 7.0%	86.9% ± 36.1%	
	⇨ Differences in SRE due to use of different PC aliquot batches.				
	Frozen raspberries (10g) Serbia (n=60)	1/500	3.0% ± 2.5%	95.2% ± 8.3%	Veg-i-Trade (in 2013)
	Company analysis IQF raspberries (10 g) (n=12)	1/50	19.2% ± 7.9%	64.3% ± 34.1%	Company X (in 2011)
	Company analysis raspberries (10g), Spain (n=3)	1/67	18.7% ± 1.6%	43.0% ± 28.4%	Organization Y (in 2011)
	Raspberries (10g) from Serbia and Poland (n=10)	1/40	12.9% ± 9.3%	46.2% ± 17.7%	Stals et al. 2011b

Figure 7.3. Picture during sample preparation of IQF raspberries as applied in Chapter 4.

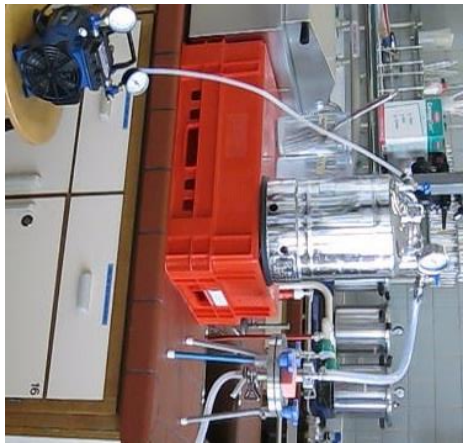


Figure 7.2. Experimental set-up of equipment for viral concentration from water used in Chapter 2.

Information needed for accurate reporting according to recent ISO/TS 15216-1 includes e.g. pLOD[1], LOQ[2] and tLOD of the method. This detailed reporting, in combination with correct interpretation of controls and a not-straight forward interpretation of the data due to current limitations of the applied molecular methods ensures that **detection of foodborne viruses is a work of specialists with relevant know-how and background, and can only be carried out in highly specialized laboratories**.

PREVALENCE & TESTING

The combination of the use of molecular detection methods such as RT-qPCR and the importance and complexity of the sample preparation step to accurately concentrate the viruses and purify the nucleic acid extracts, ensures that **NoV detection is very laborious and expensive** compared to detection of bacteria. Since the detection protocol requires expensive equipment such as centrifuges and real-time PCR equipment and the use of costly consumables and kits. This also had a repercussion on the availability of accurate data related to the natural prevalence of NoV in food and environment in non-outbreak situations, which is still rather limited in particular for leafy greens and berries (as also noted in Table 1.2. in the literature study).

During this PhD project NoV were detected in river water samples used for evaluation of viral concentration methods in **Chapter 2**. Also in the frame of the Veg-i-Trade project the methods selected during this PhD project were implemented during field studies and NoV GI were detected in 4/27 pooled lettuce samples (authenticity amplicons confirmed by sequencing), however no irrigation water samples (0/27) and hand swabs (0/21) were found positive during this open field lettuce sampling in 2012. Positive batches of frozen raspberries were identified in **Chapter 4**. These results unequivocally demonstrate the presence of NoV in fresh produce and water samples in Belgium unrelated to any known or reported foodborne outbreak. The nature of the contamination event (by e.g. contaminated hand, irrigation water) and the inability of the virus to grow outside their specific host cells, suggest that viral contamination in food products can be very low and can be very heterogeneously distributed. These observations explain at once the main

[1] pLOD, or practical limit of detection, is defined by ISO/TS 15216-1 as the lowest concentration of target in a test sample that can be reproducibly detected (95% confidence interval) under the experimental conditions specified in the method, as demonstrated by a collaborative trial or validation. NOTE the pLOD is related to the test portion, the quality or quantity of the template RNA, and the tLOD (theoretical LOD) of the method.

[2] LOQ, or limit of quantification, is defined by ISO/TS 15216-1 as the lowest concentration of target in a test sample that can be quantitatively determined with acceptable level of precision and accuracy under the experimental conditions specified in the method, as demonstrated by a collaborative trial or other validation.

limitations of currently applied batch testing regimes for detection of viruses in fresh produce for e.g. the purpose of import and export certification testing, compliance testing, and commercial supply agreements. In **Chapter 4** of this PhD thesis a multi-sample approach was applied for frozen raspberries as currently performed for batch testing of imported frozen strawberries from China. Limitations of this batch testing approach in assuring food safety and concerns of technical nature associated with RT-qPCR implementation and interpretation of results were discussed.

A *first limitation* is the **limitation of the batch testing** since viral contamination of e.g. red fruits is characterized by a heterogeneous distribution due to the nature of contamination. For example in case of contaminated irrigation water, large batches experiencing the same environmental conditions can become contaminated. Similar in case for shellfish grown in contaminated water. However in case of red fruits the fruit picker was identified in Chapter 1 as a crucial vector for enteric viral pathogens. Large batches (up to 22 tons) of e.g. IQF raspberries (limited cross-contamination events due to absence of washing) arrive that generally originate from dozens of small raspberry farms, each employing several fruit pickers. Hence illustrating the relevance of low prevalence rates in case e.g. one fruit picker was infected (pick an average of 11 kg raspberries/day). As depicted in the OC curve in Figure 7.4., in case of low prevalence of contamination the chances of rejecting this contaminated batch (=1-P(Accept)) are very slim.

E.g. in case the prevalence of NoV contaminated units is 5% in a specific batch, there is a chance of 77% that this batch will still be accepted when applying this two-class presence-absence sampling plan (n=5, c=0) and only 23% (P(reject)= 1- P(accept)) of rejecting this batch.

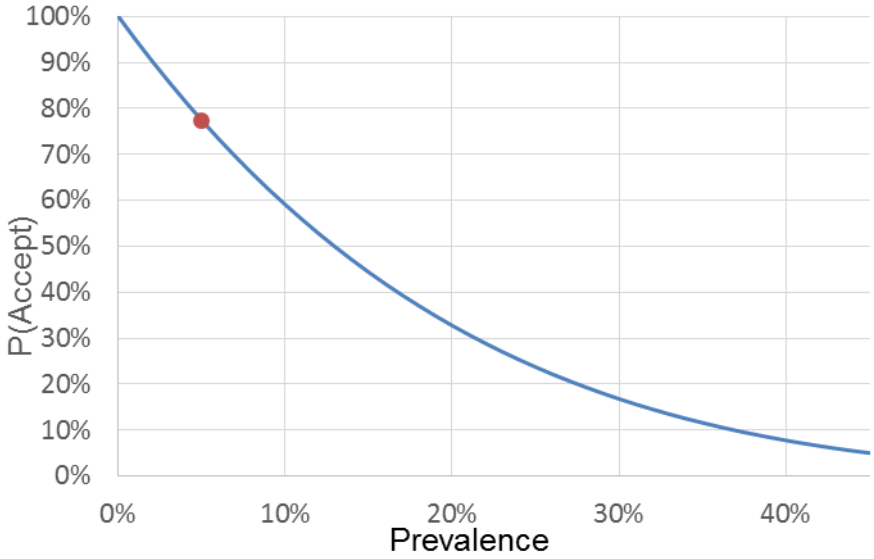


Figure 7.4. Operating Characteristic (OC) curve in case of a two-class presence-absence sampling plan defined by n=5, c=0 (Figure drawn using 'FAO MS tools.xlsm').

However insufficient data is available to have knowledge of the baseline for NoV prevalence in soft red fruits and leafy greens. Controls for NoV carried out on consignments of frozen strawberries originating from China during the period 1 January – 31 December 2013 (in EU + Norway) resulted in only two non-compliances (NoV detected) out of 98 checks (2.0%, 95% CI: [0.4, 6.4]) (EFSA BIOHAZ Panel 2014a).

A *second limitation* is the **limitation of the present detection methods**. As such current viral detection strategies are characterized by a high limit of detection (LOD) resulting in a lower sensitivity. This high LOD is due to the fractional use of RNA for molecular detection (confined by limited sample volume possible for analysis by RT-qPCR), meaning that only a fraction of the initial test matrix is eventually tested in the RT-qPCR reaction. As such, the **theoretical LOD** (tLOD) corresponds to one genome copy per volume of RNA tested in the target assay and varies according to the quantity of starting material and detection method (two-step RT-qPCR vs. one-step RT-qPCR). But also the presence of inhibitors plays a role since in case of inhibition or when amplification efficiency is <25% (as defined in ISO/TS 15216-1) the results of a ten-fold dilution of the sample RNA will be used for analysis. This results in an additional dilution of initially tested starting material during RT-qPCR and hence a ten-fold higher tLOD. As such for the raspberry analysis in Chapter 4 the tLOD mounted up to 500 GC in 10 g raspberries when 1/10 dilution of RNA samples were processed (two-step RT-qPCR) (clarified in Figure 7.5.).

Inclusion of ten-fold dilutions of RNA have also been observed as a necessity in other screening studies. As such Loutreul et al. (2014) found 12.4% (26/210) of the lettuce samples and 16% (32/200) of the red fruit samples positive for NoV. However 61.5% of the 26 NoV positive lettuce samples and 65.6% of the 32 NoV positive red fruits samples were *only* detected as positive when the ten-fold diluted RNA was analyzed. This illustrates the significant role of inhibitors in producing false negative results, especially since NoV concentrations detected in food samples are generally low and recovery efficiencies are unsatisfactory. Also in analyzed swab and water samples for Veg-i-Trade considerable inhibition was observed. Especially in case of viral detection in water samples, in literature dilutions of 1/10 to 1/100 of RNA are standard included in the analysis by RT-qPCR. As such, the presence of inhibitors limits the full potential of the possibility of analyzing large quantities of water by filtration since more inhibitors, proportional with the analyzed water volume, can be co-concentrated during sample preparation. Hence the use of larger volumes of water during viral concentration doesn't necessarily result in a higher sensitivity (Albinana-Gimenez et al. 2009).

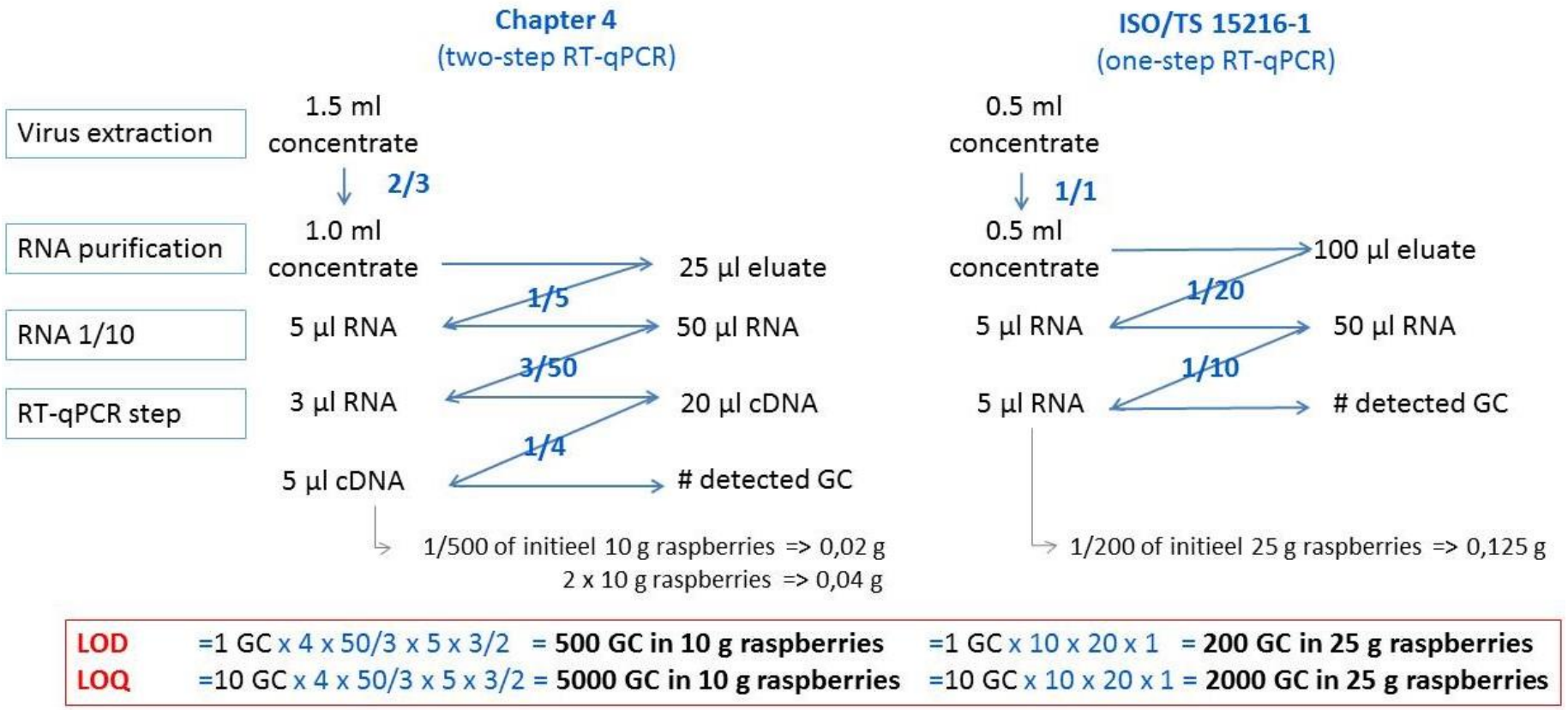


Figure 7.5. Schematical representation of fractional use of initial analytical sample unit according to the detection strategy applied in Chapter 4 (2 x 10 g analytical sample unit) and according to detection strategy as proposed by ISO/TS 15216-1 (25g analytical sample unit) for detection of enteric viruses in e.g. raspberry samples. LOD and LOQ represent here the theoretical limit of detection and theoretical limit of quantification based on in-house experience concerning pGI and pGII standard curves. Remark that LOD and LOQ are expressed per analytical sample unit. GC: genomic copies.

A second factor limiting sensitivity of present detection methods are the low sample recovery efficiencies (SRE) obtained by current methods. As such the **practical LOD** (pLOD), being the lowest concentration of target in a test sample that can be reproducible detected, is even higher than the tLOD since SRE (estimated by the use of surrogate viruses as PC) are generally low. In the ISO/TS 15216 minimal requirements are stated concerning the recovery efficiency of the PC. As such, in case where the extraction efficiency is <1%, sample results are not valid and reanalysis of the sample is required (Anonymous 2012c). Remark that a minimal SRE of 1% might seem low, but in reality this threshold is often not reached (e.g. Table 4.3.). As such SRE below 1% are not uncommon in screenings and sample analysis linked to outbreak investigations published in literature (e.g. Mäde et al. (2013) reported SRE of 0.1-1.0% in strawberries; Mattison et al. (2010) reported SRE of <0.01% in 17% the of leafy green samples), despite the detection of NoV in several samples.

In conclusion, batch testing for enteric viruses in red fruits is currently performed, however one should be vigilant of the possible heterologous distribution typical of viral contaminated red fruits and the fact that if prevalence is low the n=5, c=0 presence/absence test has a low probability in identifying a contaminated batch. Hence sampling cannot guarantee absence of NoV positive units in a batch because the sample size needed to detect low dose, low frequency, and non-uniformly distributed contamination is impractically large. Consequently the attempt to reduce the risk by sampling is often considered too little too late. However sampling of e.g. berries, irrigation water and hands can be part of a preventive approach when used for verification of GAP and GHP, when used in the frame of risk-based sampling, and when used to boost the awareness of food handlers in the farm-to-fork chain. Still if a positive signal is observed, current methods detect 'genomic copies' that are not necessarily linked to health risk, and if negative, one must be vigilant that this can indicate a true negative batch, or a false negative batch since due to limitations in sampling or limitations in sensitivity, the presence of positive sample units cannot be excluded. The sensitivity of current detection protocol is further reduced due to the typical high pLOD of current viral extraction methods. As such improvements in SRE and methods capable of dealing or removing inhibitors present in the sample RNA are needed to enable the analysis of relevant sample sizes during RT-qPCR reaction and as such limiting false negative results. Critical consideration of the appropriate sample size is also needed. As now the use of 25 g as sample size is copied from the method for detection of bacterial pathogens (e.g. 25 g portions are also analyzed to ensure absence of *Salmonella* in precut fruit and vegetables to comply with EC No 2073/2005). However due to the inability to use an enrichment step prior to detection, NoV detection differs

fundamentally from bacterial detection. Hence the current sample size of 25 g, which results in the analysis of mere 0.125 g during RT-qPCR as clarified in Figure 7.5., might be further optimized, since the use of larger sample sizes could result in the analysis of a more relevant portion.

RELEVANCE OF INDICATORS & CONTROL

A good indicator organism is a marker whose presence in given numbers points to failure to comply with applying good manufacturing and distribution practices (Ingram 1977; Mossel et al. 1995) and is hence indicative that a food has been exposed to conditions that pose an increased risk that the food may be contaminated with a pathogen or held under conditions conducive for pathogen growth (IFT/FDA 2001). Overall *E. coli* is considered to be an adequate bacterial indicator of fecal contamination. As such *E. coli* is used in the legal framework as a process hygiene criteria in relation to pre-cut ready-to-eat fruits and vegetables (n=5, c=2, m=100 CFU/g, M=1000 CFU/g; EC No 2073/2005). Also for assessment of suitability of irrigation water sources, historical microbiological criteria are based on the presence of e.g. *E. coli* or fecal coliforms. Currently available guidelines regarding the quality of water used in primary production have been reviewed recently by Holvoet (2014). However in case of other pathogenic micro-organisms such as enteric viruses and protozoa, bacterial indicators such as *E. coli* generally fail to signal the potential for viral or protozoan contamination (discussed in **Chapter 6**) as bacteria are expected to decrease/degrade quicker than the more stable viruses in adverse conditions outside of the animal/human reservoir. This was also observed during the batch screening in **Chapter 4**, since all batches were considered to be of good microbiological quality (due to absence of *E. coli*), despite presence of NoV in a subset of batches.

For viral contamination, bacteriophages, enteroviruses, picobirnaviruses, polyomaviruses and adenoviruses have all been proposed as alternative viral indicators for the presence of *human* fecal contamination (Savichtcheva and Okabe 2006; Symonds et al. 2009). Especially **human adenoviruses** (hAdV) have been recommended as a potential indicator of human fecal pollution and a virological index for water quality since they persist wastewater treatment well, have been shown to have greater stability than enteroviruses, and are present more consistently in the environment than NoV and enteroviruses (Carter 2005; Charles et al. 2009; Silva et al. 2011). As such hAdV have been detected at similar (high) rates in diarrheal (44%) and non-diarrheal (53%) specimens in a wide range of age groups, from a few months old to over 80 years of age (Mans et al. 2014). While NoV GI and GII were detected in that same study in 0% and 7% respectively in diarrheal specimens (n=94) and 1% and 22% respectively in non-diarrheal specimens (n=93) (Mans et al.

2014). However, despite the availability of a whole body of literature concerning the presence of hAdV in water matrices (Carducci et al. 2009; Wyn-Jones et al. 2011) and shellfish (Muniain-Mujika et al. 2003; Pina et al. 1998), consistent sampling studies that study hAdV in combination with NoV in the fresh produce chain are lacking. Hence more data are required before suitability of an enteric viral indicator can be validated (EFSA BIOHAZ Panel 2014a) and ideally candidate viral indicators should be included in future baseline studies.

Concerning mitigation strategies for control of foodborne viruses such as NoV in the fresh produce chain, control options can be divided in two categories: (i) **prevention actions** limiting the entry of NoV in the farm-to-fork chain, and (ii) **intervention actions** reducing viral contamination by inactivation/removal of the viral load during processing. The main mitigation strategies identified in this PhD thesis for both categories will be discussed briefly. For more detailed information concerning mitigation strategies for NoV in the fresh produce chain the following relevant documents can be consulted online:

- CAC/GL 79-2012: Guidelines on the application of general principles of food hygiene to the control of viruses in food, with Annex II on Control of hepatitis A virus (HAV) and norovirus (NoV) in fresh produce.
- EFSA BIOHAZ Panel, 2014. Scientific opinion on the risk posed by pathogens in food of non-animal origin. Part 2.
    - *Salmonella* and Norovirus in leafy greens eaten raw as salads. EFSA Journal 2014; 12(3):3600, 118 pp. doi: 10.2903/j.efsa.2014.3600
    - *Salmonella* and Norovirus in berries. EFSA Journal 2014; 12(6):3706, 95 pp. doi: 10.2903/j.efsa.2014.3706
- VITAL guidance sheets on enteric viruses in the farm-to-fork supply chain of berry fruits and leafy greens. Available online at eurovital.org/index.htm

PREVENTIVE ACTIONS AS MITIGATION STRATEGIES

As stated in EC 2073/2004, the safety of foodstuffs is mainly ensured by a preventive approach, such as implementation of Good Hygiene Practices (GHP) and application of procedures based on hazard analysis and critical control point (HACCP) principles. Hence in order to select valid prevention actions, a thorough knowledge of the whole farm-to-fork chain is required. Therefore in **Chapter 1** of this PhD thesis a literature study was undertaken that includes an elaborate section on identified viral transmission routes in the fresh produce chain (§1.2.). The main transmission routes identified in Chapter 1 are contaminated food handlers and water.

Contaminated hands and surfaces play an important role in the transfer of viral enteric pathogens such as NoV. As such knowledge and availability of proper hand washing and drying practices, food handling practices (e.g. food handling while sick), and correct handling and cleaning practices in case of contamination (e.g. by vomiting) is crucial among food handlers and food safety professionals. Nevertheless current awareness and knowledge on NoV was found lacking among food handlers and food safety professionals assessed during surveys performed respectively in the Netherlands (Verhoef et al. 2013) and in the USA (Kosa et al. 2014). Gloving is often proposed as a solution to prevent viral transfer from pickers and food handlers, but remark that gloving in absence of good hand hygiene can still result in viral transfer. This was experimentally demonstrated in a recent study by Rönnqvist et al. (2014) in which transfer of MNV-1 dried on the hands during gloving was observed 10/12 times. Hence stimulating an appropriate food safety culture in which compliance to hygienic standards is encouraged and pursued day after day is crucial.

Despite the current limitations of testing used in case of compliance testing, testing can also be part of a preventive approach by sampling of the production and processing environment (including hands) as tool to identify and prevent the presence of pathogenic micro-organisms in foodstuffs (EC 2073/2004). Surfaces can be more easily monitored in function of a food safety management system than the food itself since higher recoveries, less inhibitors and an easier protocol are characteristic of the swab protocol. This way **testing is used as process verification**, to check the status of hands, surfaces, and the application of GHP, and can have a huge positive impact on the behavior and awareness of food handlers. Also **risk-based sampling** of *hands*/surfaces, but also of *products* and *water* during e.g. prerequisite compliance audits, where epidemiological studies indicated a higher risk of infection or at the discretion of the food business operator, can be part of a holistic approach for food safety assurance (EFSA BIOHAZ Panel 2014a).

Contaminated water, either in the role of irrigation water or process water, can also play an important role in the transfer of enteric viruses. In **Chapter 5** the role of processing water as a (cross-)contamination pathway during a simulation of an industrial wash process of lettuce was explored. This study illustrated that the water quality should be maintained at an acceptable level during the whole washing process and stresses the importance of the inclusion of an effective sanitizer to prevent cross-contamination. To summarize current knowledge on water as a transmission route and possible mitigation strategies, a review was performed in **Chapter 6** on the available QMRA studies in peer-reviewed literature that included the modeling of effect of water use (e.g. influence of irrigation water and/or

washing step) or water treatment on the quality of fresh produce in at least one stage of the farm-to-fork supply chain. Identified preventive actions related to the use of water include e.g. the selection of an appropriate water source, performance of water treatment, choice of irrigation method, crop selection, and inclusion of a withholding period. Also minor adaptation of the currently implemented systems could improve water quality. As such, presently used irrigation water treatment in e.g. lettuce production primarily focus on elimination of phytopathogens. However additional assessment and improvement of the effect of treatment regarding foodborne pathogenic bacteria and enteric viruses is relevant and could result in an additional mitigation step requiring only minor adaptations to the system in place.

Regular **testing of irrigation water sources** for the presence of relevant indicators and if necessary pathogens is also conform good agricultural practices (GAP). As elucidated in Chapter 6, when different reference pathogens were compared, the health risk for enteric virus infection by contaminated fresh produce due to contaminated irrigation water was higher compared to infection risk by other reference pathogens as *Campylobacter* and *Cryptosporidium*. The high health risk is a result of the recalcitrance of enteric viruses to water treatment, their high environmental persistence, and especially because of their low infectious dose. However the currently used fecal indicator *E. coli* in irrigation water does not suffice as indicator for viral risks if fecal contamination is from human origin. In contrast to viruses, *E. coli* does not have a narrow host range and hence cannot be used as a specific indicator for *human* fecal contamination. Additionally, due to specific characteristics and high recalcitrance of enteric viruses, viruses such as NoV could persist longer than *E. coli* in certain conditions. However, direct pathogen detection in (relevant volumes of) water is expensive. In case of source water for drinking water production, large volumes (e.g. 200 to 600 L in Lodder et al. (2010)) of the water are to be tested for the presence of enteric viruses. However in this branch the substantial costs of sampling and analysis are accepted since drinking water is overall strictly controlled as contamination would have huge impacts on public health. However these costs would be difficult to accept in case every farmer would have to check their irrigation water following the same standards as for source water for drinking water production. Anyhow, in a QMRA study of Stine et al. (2005b) a maximum allowable concentration for HAV in irrigation water for fresh produce (applying a worst-case scenario: consumption one day after irrigation) to ensure an annual risk of ≤1:10 000 was calculated to be 2.5×10^{-5} most probable number per 100 mL in case of furrow irrigation of lettuce. The analysis of 1-10 L water as aimed for in Chapter 2 would hence not suffice to check if this limit is exceeded. However major non-compliances could be detected if 1-10 L is analyzed. Also microbial

source tracking methods to identify if human fecal contamination is present could be relevant using these lower volumes. Direct measurement of viral pathogens and bacteriophages with different host groups, e.g., hAdV, bovine polyomavirus (bPyV), porcine adenoviruses (pAdV), and the F+ RNA coliphages have been suggested as an interesting, library-independent **microbial source tracking method** to identify the origin of contamination in *aquatic* environments (Fong and Lipp 2005). However further validation at farm-level and along the farm-to-fork chain of fresh produce is requested to be able to assess the true value of this tool. Anyway, risk evaluation concerning the possible contamination sources of the irrigation water used and preventive actions such as chlorination or UV treatment of the irrigation water should be the basic pillars in preventing NoV contamination via contaminated water. As nowadays implemented sampling frequency of once a year fails to detect e.g. the natural variability during seasons and sporadic unexpected failure of water treatment. Also when the water is according to the risk evaluation not subjected to fecal contamination of human origin, testing for specific indicators of *human* fecal contamination that can be a marker for the presence of enteric viruses should be encouraged in case of extreme events (e.g. heavy rain, flooding, etc.).

For further discussion on the quality and testing of water used in agriculture or during processing I refer to recent PhD dissertations accomplished in collaboration with the LFMFP lab in the frame of the European Veg-i-Trade project (i.e. Holvoet 2014; Van Haute 2014).

INTERVENING ACTIONS AS MITIGATION STRATEGY

Some food preparation measures, as reviewed in **Chapter 1**, do have a positive impact on the viral load and hence can be included in the farm-to-fork chain as a mitigation strategy. Though the **primary focus should be on prevention**, since once contamination of fresh produce has occurred subsequent frequently used steps in industry that allow the retention of fresh-like organoleptic properties (e.g. washing) will not result in total elimination of foodborne (viral) pathogens. As such, washing only results in maximum 1-2 $\log_{10}$ reduction in viral load. The main effect of sanitizers during washing of fresh-cut produce is aimed at reducing and controlling the microbial load of the water used in fresh-cut processing and thus prevent cross-contamination, rather than having a decontamination or preservative effect on the produce itself. Hence it is important to study the effectiveness of a sanitizer not only on the produce, but also on the viral load in the resulting wash water. However the latter is not always included in experimental studies in literature. In a recent inactivation study using a porcine gastric mucin binding magnetic bead (PGM-MB) assay

in combination with RT-qPCR to determine the effectiveness of several commonly used chemical sanitizers on human NoV, the use of chlorine (sodium hypochlorite) was advocated for use as a human NoV disinfectant wherever possible (sodium hypochlorite, 189 ppm free chlorine (FC) resulted in 4 $\log_{10}$ reduction). As remarkable resistance was noted of human NoV (in 10% stool filtrate) towards the other tested chemical sanitizers such as chlorine dioxide, peroxyacetic acid, hydrogen peroxide, and trisodium phosphate (Kingsley et al. 2014).

To prevent large outbreaks due to virally contaminated frozen red berries especially large-scale-kitchens should strictly adhere to the instruction to **heat the berries before serving**. However, as identified in the literature study, data concerning heat treatments of produce at temperatures >75°C are scarce. Hence additional relevant heat inactivation studies for this high temperature range in relevant produce matrices and for several (surrogate) viruses should be conducted to obtain more insight. However, inactivation studies using molecular-based methods for estimating capsid integrity (using RNase or propidium monoazide) suggest that human NoV might be more heat resistant than common cultivable surrogate viruses (Escudero-Abarca et al. 2014). Hence ideally confirmation and validation of the effectiveness of recent recommendations for inactivation of NoV by heating of soft ref fruits (e.g. 90°C for 90s, or heating of frozen raspberries for 2 min at 90°C as stated on the package of raspberries (Figure 1.12.) in Chapter 1) should be accomplished. However not ideal, human feeding trials - such as recently performed for evaluating the effect of HPP on human NoV inactivation in oyster (Leon et al. 2011) - could serve for this purpose.

Assigning absolute risk reduction values to measures used in the mitigation of risk is challenging due to uncertainty in the effect on enteric viruses such as NoV due to use of surrogate viruses in persistence/removal/inactivation experiments. But it is also challenging due to the sporadic nature of pathogen occurrence and localized conditions leading to the persistence of pathogens in the environment. However there is a large body of evidence showing that human NoV are both more persistent and more resistant than surrogate viruses. This is strengthened by a recent qualitative meta-analysis that showed that human NoV appear more persistent than surrogate FCV or MNV in response to heat and available chlorine than previously recognized (Knight et al. 2014). As such impact of certain mitigation steps using QMRA is estimated using surrogate data, but the exact significance of impact on human health risk concerning the pathogen under study (i.e. NoV) remains largely uncertain.

7.3. CONCLUSIONS AND RECOMMENDATIONS

Five years after the start of my research on foodborne viruses, the topic is still highly relevant and challenging. However despite the continuous effort the last 15 years concerning NoV detection and data gathering for matrices such as fresh produce, a lot of questions remain to be solved today. This was also perceived during this PhD project as when attempting to analyze NoV transmission and presence in the food-chain of fresh produce a lot of uncertainties concerning NoV add up as specified in previous paragraph (i.e. §7.2).

The unavailability of an appropriate cell culture assays for foodborne viruses such as NoV has resulted in the use of molecular detection methods and the use of surrogate viruses for persistence and inactivation studies. This has set a cascade of resulting question marks and challenges in motion concerning related health risk associated with the presence of genomic copies (GC), effect of risk mitigation strategies, and has put pressure on the efficiency of the extraction protocol in order to lower the LOD due to the inability of performing an enrichment step. Hence the search for an appropriate validated cell culture assay for NoV should continue.

OPINION ON ESTABLISHMENT OF MICROBIOLOGICAL CRITERIA AND PREREQUISITES

More studies are needed on the **relation between detection of virus genomic copies by PCR in food and the relevance for human health**. As such, valuable information could be achieved by **further data gathering** (baseline data) and better reporting on quantities or order of magnitude of NoV genomic copies detected in outbreak situations. The availability of such data could allow meta-analysis of quantitative results obtained for NoV RNA levels in berries associated with illnesses and RNA levels in non-outbreak-related samples. This to better know what a signal means and to have an indication of reason for concern (in non-outbreak situations). Such a study was performed by Lowther et al. (2012) for oysters in which a significant difference was found between NoV levels detected in oyster samples strongly linked to NoV or NoV-type illness (geometric mean: 1048 GC/g), and levels typically found in commercial production areas (non-outbreak-related samples, geometric mean: 121 GC/g). In case of the problem of NoV in shellfish, these observations supported the concept of a dose-response for NoV RNA levels in shellfish and valuable data were obtained to inform the development of robust risk management criteria.

As such, establishment of **microbiological criteria** has been recommended for NoV presence in oysters (EFSA BIOHAZ Panel 2012). However for fresh produce, prevalence studies and quantitative data on viral load are scarce making European consensus for establishment of microbiological criteria for these food categories difficult (EFSA BIOHAZ Panel 2011, 2014a). Furthermore, detection of NoV in shellfish has several advantages over detection of NoV in fresh produce. As such, when in our lab data related to the efficiency of viral extraction and concentration protocols for shellfish and fresh produce matrices are compared, the sample recovery efficiency is generally much higher in case of shellfish (50% of SRE ≥25% in Li et al. (2014b)) compared to the SRE obtained for fresh produce matrices (Table 7.1.). Furthermore, NoV contamination of fresh produce consist most probable of low numbers of heterogeneously distributed viruses, while in shellfish extraction is further optimized by focusing on the digestive glands that are known to accumulate NoV particles during the filter feeding process. Finally, for NoV detection in shellfish it has been suggested that shellfish detection methods mostly detect intact particles since bioaccumulation of RNA in shellfish appears to be insignificant in an experiment by Dancer et al. (2010). This possibly increases the relevance of detection of viral RNA in shellfish related to associated public health risk. However further verification of this is needed (Butot et al. 2014). As such, the fact that the ratio infectious virus particles: number of viral GC detected by PCR may vary according to environmental conditions, history of treatments (e.g. heating, HPP) and 'age' of the contamination, especially for food products other than shellfish, further challenges the establishment of an overall European consensus on a threshold infectivity limit for NoV detected by RT-qPCR (EFSA BIOHAZ Panel 2012). Hence modified detection protocols that could establish a better link with human health risk are ideally required to enable unambiguous interpretation. Other **prerequisites for establishment of a (quantitative) limit for presence of NoV** is further method validation, improvement in sample preparation, practical LOD, and improvement in 'quantitative' accuracy as noted during recent EU reference laboratory proficiency test (PT 46) (Cefas 2013). Furthermore, microbiological criteria have been suggested to be less effective at managing risk when low levels of contamination are considered (limitation of batch testing), which is the most likely situation in case of NoV in fresh produce. Remark also that if a microbiological criteria for NoV in fresh produce would be implemented now, with the current limitations of the detection strategy and the inherent limitation of batch testing, the *consumer's risk*, i.e. the chance that a batch will be accepted that exceeds the limit and hence pose an unacceptable risk to public health, will be considerable.

Despite the lack of a correlation between infectivity and the presence of GC, RT-PCR detection indicates unequivocally contact with the considered viral pathogen and hence a breach of hygienic practices. However microbiological criteria should be scientifically based, hence accurate **baseline data is necessary** to have an objective overview of the situation and to have an idea of the relevance of any sampling plan. Baseline data is also needed to estimate the impact of a certain criteria (e.g. number of corrective actions that would be needed, benefit for public health) as establishment of a specific microbiological criterion is a risk management decision and requires thorough insight into the issue. As such, in the case of NoV presence in shellfish microbiological criteria suggested are based on a certain threshold instead of presence-absence testing since quantitative data on viral load from areas compliant with current EU legislative requirements (*E. coli* standards) during January-March 2010 in the UK, Ireland and France, show that a NoV action limit of 100, 200, 500, 1000 or 10,000 NoV PCR genome copies would result in 33.6-88.9%, 24.4-83.3%, 10.0-72.2%, 7.7-44.4% or 0-11.1% of non-compliant batches, respectively (EFSA BIOHAZ Panel 2012). Hence in shellfish a standard based on NoV absence would have a high impact, while presence of NoV positive signals in oysters were not necessarily linked with outbreaks in a study by Lowther et al. (2010, 2012).

The absence of microbiological criteria for NoV on European level and the limitations of current detection strategy as discussed in paragraph 7.2. (high LOD, interpretation, need for standardization) should however not result in ignoring the potential risk of viral contamination in fresh produce matrices. As when NoV genomic copies are present, risk for human health cannot be excluded, unless the sample history (e.g. thorough heat treatment) can prove otherwise. However, even in the latter case, unhygienic practices are linked to presence of NoV GC and hence appropriate actions are needed depending on local risk management decisions. Note that sampling and testing always have its limitations, whether for detection of the presence of NoV or other bacterial pathogens, e.g. shiga toxin-producing *E. coli* (Delbeke 2015). Local authorities can stimulate the awareness of local food producer/processing companies by active sampling and analysis for presence of NoV in food products and to communicate on these results and actions taken. This could highlight the point that presence of NoV (or NoV GC indicating historical human fecal contamination) in food (or irrigation/processing waters or food contact surfaces) is not acceptable.

If one decides upon setting up a sampling, in particular risk based sampling could be considered. This risk based sampling can be driven by knowledge obtained either during an inspection or audit revealing (systematic) noncompliance on good hygienic practices, or

during a sanitary survey indicating risk factors for (human) fecal contamination, or having notification of reported illness or epidemiological evidence available to the discretion of the food business operator or control authority indicating a higher risk of NoV contamination (EFSA BIOHAZ Panel, 2014a). The latter resulted in import control for presence of NoV and HAV on frozen strawberries from China in 2013 (Anonymous 2012a) as a result from the German outbreak in 2012 (Mäde et al. 2013).

Relevance of an ISO method and points needing further improvement

The availability of an ISO method for the extraction, concentration and detection of NoV in the food chain, despites it imperfections, is an enormous step forward. As it provides a framework for the correct implementation and use of necessary controls during extraction and detection steps, and now new methods can be unambiguously compared to the ISO/TS 15216-1 (e.g. in Coudray et al. 2013). Detection of viruses is laborious and significantly different from standard bacteriological analysis and hence requires a well-equipped lab and intensive training of lab technicians. Further capacity building is needed by (preferably) interlaboratory validation studies or collaborative trials. In addition effort is needed to establish availability of **reliable quality control materials.** This in particular being a prerequisite for **further standardization** of the **quantification step**. Concerning the detection strategy, the **sample processing step** has been identified as a crucial, but to date the **limiting factor** for detection of enteric viruses in fresh produce and water samples. This is due to high variability of virus recoveries and the low extraction efficiency. Despite the initial acceptable recovery efficiencies obtained during comparisons in research and method development, in practice the obtained recovery efficiencies have been observed to be much lower (e.g. in Mäde et al. 2013).

Recommendations concerning mitigation strategies

Controlling viruses in fresh produce clearly needs a food-chain approach in which **prevention measures** to avoid viral contamination should be the central strategy rather than trying to remove/inactivate viruses from/on fresh produce. Although use of an effective **sanitizers** during industrial fresh produce washing is a necessity **to limit cross-contamination** events. Despite the current absence of specific EC legislation concerning microbiological criteria for viruses in fresh produce it should be clarified that infectious NoV particles should be absent in food and that implementation of good hygienic, agricultural and processing practices along the farm-to-fork chain should be the main objective since the latter is crucial for limiting viral threats. Adequate **guidelines concerning** appropriate water treatment or **water quality** of water to be used for fresh

produce irrigation should strive for taking into account also the viral threat. These requirements should preferably be set based on risk assessment. However limitations of current QMRA concerning water in the fresh produce chain have been discussed in Chapter 6, and problems are associated with feasibility, the meaning of indicator detection for viruses and cost of monitoring (EFSA BIOHAZ Panel 2011). Direct pathogen detection is costly, but **further research** concerning **microbial source tracking** and validation of its use at farm-level would be interesting.

As a final critical note, the importance of the behavior of food handlers in respecting good hygienic practices and hand washing should once more be highlighted. Food handlers hygiene was reported as a contributing factor in NoV contamination in 70% of NoV FBO reporting contributing factors in the USA (2009-2012) (Hall et al. 2014). Hence an important issue for controlling viral contamination is by proper **education and incitement of** involved **food handlers**. The establishment of a proper **food safety culture** in which compliance to hygienic standard is pursued day after day is crucial. Again local authorities can play an important role in this by providing information and building awareness by means of sampling and testing for the presence of NoV on relevant surfaces and hands in food production areas (Verhoef et al. 2013). As such outreach and education are also an important part of the huge USA NoroCORE project. Online more and more information is available but also at the farm-level food handlers such as pickers should be properly instructed and necessary facilities (e.g. toilet and proper hand wash facilities) should be present within reach. Since hand hygiene is the most critical control point in preventing viral contamination of specific fresh produce products (e.g. red fruits) and viral FBO in general.

7.4. PERSPECTIVES FOR FURTHER RESEARCH

Next to further research touching the relation between detection of virus genomic copies by PCR in food and the relevance for human health (including baseline studies), future research concerning NoV in fresh produce should focus on:

1) Limitations and weaknesses of current detection strategy

To limit false negative results and to improve the true value of a negative result for the presence of NoV in a fresh produce sample future research should aim to **lower the practical LOD**. As now 'absence' in 25 g fresh produce means practically 'possible presence of NoV below pLOD' which is as high as 2-3 $\log_{10}$ GC per sample. As such further improvements are needed concerning the low sample recovery efficiency (SRE) and the inability of the current detection strategy to adequately deal with inhibitors. For this the use of an automated system based on cationic separation or immunoconcentration, such as the Pathatrix have the potential to more specifically concentrate the viruses and to allow a 'washing step' before further RNA extraction which has the potential to remove inhibitors. Also the small actual sample volume analyzed during RT-qPCR, as schematically depicted in Figure 7.5., should be addressed. As such questioning the use of 25 g as appropriate sample volume is needed. Again the use of an automated system using cationic separation such as the Pathatrix has the capacity to potentially analyze the higher amounts of elution buffer possibly needed to analyze higher volumes of fresh produce samples without requiring too much additional hand-work.

Further standardization of reliable quality control materials is needed in order to be able to benchmark the results obtained in different labs. In Chapter 4 a hypothesis was suggested that the quality of the process control batch (difference in virus particles to genomic copies ratio) could lead to heterogeneity of obtained SRE. This hypothesis could be further investigated by better characterization of the inoculum (e.g. MNV-1) using cell-culture, RT-qPCR and possibly by a cell-binding detection assay and then performing the extraction from the raspberries and characterize the eluted and concentrated viruses again by cell-culture, RT-qPCR and possibly by a cell-binding detection assay. This allows the calculation of the 'recovery efficiency' using these three different detection options to have an idea if viral particles are more efficiently extracted and concentrated compared to naked viral genomes. A similar experiment has been performed for the detection of poliovirus in water by Haramoto et al. (2007).

2) Practical control and prevention at farm-level

Further evaluation of human adenoviruses (hAdV) as **enteric viral indicator** during the farm-to-fork chain of fresh produce is needed. Ideally this candidate viral indicator should be included in future baseline studies. Also information of the presence of hAdV in batches

of fresh produce linked to viral FBO would help to study the relevance of this potential indicator in assessing viral risk for contamination with NoV or HAV. Studies on the presence of hAdV in the farm-to-fork chain of fresh produce are rare. In the VITAL project data on the presence of the index virus hAdV and of viral pathogen such as NoV and HAV was collected in the European berry fruit supply chain (Maunula et al. 2013) and the European leafy green vegetable supply chain (Kokkinos et al. 2012). However not all samples tested for presence of hAdV were consistently checked for the presence of NoV and HAV. As such more consistent sampling and data are needed to have a clear insight of the use of hAdV as potential indicator of human fecal pollution. As the implementation of sampling of an 'indicator' virus has been suggested as a tool to build trust in certain supply chains (Butot et al. 2014).

Microbial source tracking of fecal pollution in irrigation waters is a possible strategy to allow risk based decision making and implementation of risk based control options. Since direct pathogen detection of NoV and HAV is costly and practical volumes of 1-10 L might not suffice to give an indication of the viral risk of the irrigation water. However analysis of these relative 'small' volumes of 1-10 L might be relevant for source tracking purposes using viral pathogens and bacteriophages with different host groups. The use of microbial source tracking (MST) methods to identify the origin of contamination in aquatic environments has currently primarily focused on environmental waters (e.g. in Lee et al. (2014)), recreational and bathing waters (e.g. in Staley et al. (2012)), and shellfish harvesting areas (e.g. in Ma et al. (2011), Mauffret et al. (2012)) and shellfish (e.g. in Mieszkin et al. (2013)). As such practical evaluation of this strategy for assessing viral risk concerning the use of different types of irrigation water for irrigating fresh produce consumed raw, and for situations that can impact these waters such as heavy rain, run-off water, flooding, temporarily breakdown of wastewater treatment, is needed. Because if human sewage can contaminate this water, *E. coli* as an indicator of fecal contamination may not suffice to assess the viral risk for public health inherent to the water source. As such screening of irrigation waters under different circumstances (e.g. different seasons, flooding, run-off, breakdown WWTP, heavy rain) should be performed at farm-level for the presence of HAV and NoV in relevant volumes and these outcomes should be compared to the risk assessment following microbial source tracking in 1-10 L irrigation water samples. An added advantage is that some of the most frequently used viruses for microbial source tracking, such as hAdV and F+ RNA coliphages are culturable and hence can be used to assess the impact of local water treatments implemented as mitigation strategy.

SUMMARY - SAMENVATTING

SUMMARY

Chapter 1 presents a literature overview with respect to foodborne viruses such as norovirus (NoV) and hepatitis A virus (HAV) in the fresh produce chain. A short introduction on foodborne viruses, with the focus on NoV, was presented. As such a brief history of NoV as foodborne pathogen, typical (disease) characteristics, and detection methods for food- and waterborne viruses were summarized. Furthermore, the relevance of NoV as a foodborne viral pathogen in fresh produce was illustrated in an outbreak table (Table 1.1) that described foodborne outbreaks (FBO) due to the two most frequently linked viruses with FBO, namely NoV and HAV. This table contains peer-reviewed outbreak investigation studies that date back till 1979 and presents epidemiologic evidence for the relevance of the two chosen case studies in this PhD project: i.e. NoV and lettuce (leafy greens), and NoV and raspberries (red fruits). In a second and a third part of this literature overview the transmission routes relevant for fresh produce, the environmental persistence, and the effect of treatments use in food processing of fresh produce on the viral load were summarized. Since information on each of these topics is vital for identification of effective prevention, control, and intervention strategies for risk mitigation. As such, both contaminated food handlers and irrigation/processing water were identified as relevant transmission routes for enteric viruses in the fresh produce chain. In contrast to the overwhelming body of literature demonstrating the importance of the food handler, limited studies have focused on the importance of water, especially processing water as a possible route of contamination with enteric viruses. In case of contamination of fresh produce, NoV will persist between contamination and consumption due to their high environmental persistence, and their high resistance to commonly used decontamination practices (e.g. washing) for fresh produce. This illustrates the importance of a preventive approach for risk mitigation.

In **Chapter 2** and **Chapter 3**, the in-house methods to enable detection of NoV in the farm-to-fork chain were completed with a selected method for detection in irrigation and processing water and a method for detection of NoV on surfaces and hands. As such in **Chapter 2** four viral concentration methods were evaluated for their efficiency in recovering murine norovirus-1 (MNV-1) and MS2 bacteriophages from processing water (1 L) and four different types of irrigation water (bore hole water, rain water, open well water and river water) (2-5 L). The best option was ‘Method 1’, a method based on the VIRADEL principle using an electronegative HA-filter in combination with an alkaline elution buffer based on Triton X-100 and a secondary concentration method based on precipitation with PEG. Further evaluation was performed of this Method 1 for the detection of human enteric viruses HAV, GI and GII NoV, and rotavirus (RV) in the five previous water types. In conclusion, Method 1 proved to be a reliable method for detection

of HAV, GI and GII NoV. However, for detection of RV the method proved less effective as significant lower sample recovery efficiencies (SRE) were obtained for RV in all water matrices tested (SRE <2%).

In **Chapter 3** three different strategies for swab elution/extraction, including one strategy based on the recently launched ISO protocol (ISO/TS 15216-1), were compared to optimize the swabbing methodology for detection of viruses on (food) contact surfaces. As such an improvement is proposed to the currently applied ISO/TS protocol for detection of NoV and HAV from surfaces, since sample recovery efficiencies (SREs) obtained with the new semi-direct lysis strategy (strategy 3) were significantly higher. Further comparison of cotton swabs, polyester swabs and biowipes (Biomérieux) for detection of NoV GI and GII inoculated on HD-PE, neoprene rubber and nitrile gloves, identified biowipes as the best option for detection on the coarser neoprene rubber. No significant difference was observed in the SREs obtained for the other two surfaces.

In **Chapter 4** batch testing for NoV (GI & GII) was performed on 14 batches of frozen bulk raspberries intended for the production of puree and on 12 batches of IQF raspberries. The batch testing regime currently used for import control of frozen strawberries from China (n=5, c=0) was applied and a decision tree was elaborated to support interpretation of NoV RT-qPCR results. The added value of an additional confirmation of positive RT-qPCR signals by sequencing of the resulting amplicon was demonstrated since false positive batches, even though all implemented controls were negative, were identified. The present study demonstrated the presence of quantifiable amounts of NoV clustered in several positive samples in one batch, indicating sanitary problems. Furthermore the limitations of batch testing and current detection methods for NoV in fresh produce were discussed. As such it remains the question whether batch testing is cost-effective and the most appropriate manner to guarantee food safety, since sampling cannot guarantee absence of NoV positive units in a batch because the sample size needed to detect low dose, low frequency, and non-uniformly distributed contamination is impractically large. Furthermore, the standard inclusion of a process control (PC) and the standard reporting of these data is encouraged, however additional research was requested to improve the availability of reliable quality control materials.

In **Chapter 5** and **Chapter 6** additional information was gathered on the potential role of processing and irrigation water as contamination source of enteric viruses in the fresh produce chain. The study in **Chapter 5** focused on processing water as potential source for cross-contamination and collected quantitative data using a dynamic two-step washing process (sanitizers not included) as a simulation of a commercial lettuce washing process. Two cross-contamination processes (from lettuce to water and from water to lettuce) were

included in the simulation study and the transfer of *E. coli*, *E. coli* O157, MS2 phage and MNV-1 was quantified. The transfer of these microorganisms from water to lettuce, was determined by contaminating washing bath 1(WB1) with three different inoculation levels. Besides the cross-contamination between the water in both WBs, the non-contaminated incoming lettuce was also contaminated through contact with the contaminated water from WB1. Transfer ratios, quantifying the cross-contamination of microorganisms from the inoculated WB1 to the lettuce ranged overall from 0.3% to 1.5%. The quantitative data of lettuce contamination and transfers established in this study further highlight the vulnerability of fresh produce to cross-contamination and illustrates the necessity of the use of appropriate sanitizers to limit the possibility for cross-contamination.

The aim of **Chapter 6** was to review peer-reviewed quantitative microbial risk assessment (QMRA) studies on water and safety of fresh produce to develop a holistic assessment from source water to farm-to-fork chain and where water is either included as potential vehicle for foodborne pathogens. The availability of site-specific data was identified as one of the most recurring data gaps. Specific parameters lacking hard data included pathogen penetration in food crops; pathogen survival/persistence on or in food crops, and rates of pathogen transfer from irrigation water, and other water sources (e.g. processing water), to crops. Hence illustrating the relevance of the research in Chapter 5.

The knowledge acquired in the above-mentioned chapters have led to the formulation of general conclusions in **Chapter 7**. Furthermore Chapter 7 includes the discussion of the uncertainties related to health risk, standardized detection, prevalence and testing, and relevance of indicators and control encountered when you strive for a holistic understanding of NoV in the farm-to-fork chain. Perspectives for further research were announced.

SAMENVATTING

Hoofdstuk 1 geeft een overzicht van de literatuur met betrekking tot voedsel-gebonden virussen zoals NoV en HAV in de groenten & fruit (G&F) productieketen. Een korte inleiding en geschiedenis van NoV als voedsel-gebonden pathogeen werd aangevuld met de typische karakteristieken en detectiemethoden voor voedsel- en watergebonden virussen. Verder werd de relevantie van NoV als voedsel-gebonden pathogeen in G&F geïllustreerd aan de hand van een tabel met uitbraken te wijten aan de twee belangrijkste voedsel-gebonden virussen: NoV en HAV (Tabel 1.1.). Deze tabel bevat uitbraakonderzoek studies die terug gaan tot 1979 en leveren epidemiologisch bewijs voor de relevantie van de twee gekozen case studies in dit doctoraat: nl. NoV en sla (bladgroenten) enerzijds en NoV en frambozen (rode vruchten) anderzijds. In een tweede en derde deel van deze literatuur studie werden de transmissie routes relevant voor G&F, de overleving, en het effect van behandelingen frequent gebruikt tijdens de processing van G&F op de virussen samengevat. Daar informatie over elk van deze drie onderwerpen cruciaal is voor de identificatie van effectieve preventie, controle, en interventiestrategieën ter beheersing van het risico gelinkt aan NoV in G&F. Zowel voedselbehandelaars als irrigatie/processing water zijn geïdentificeerd als relevante transmissie routes voor voedsel-gebonden virussen in de G&F keten. Maar in tegenstelling tot de grote hoeveelheid uitbraakstudies en experimentele studies voorhanden die het belang omschrijven van de voedselbehandelaar, zijn slechts een beperkt aantal studies beschikbaar die focussen op het belang van water (vooral beperkt voor waswater) in de transmissie van voedsel-gebonden virussen in de G&F keten. In geval van contaminatie van G&F, zorgt de hoge overlevingsgraad van NoV in de omgeving ervoor, gecombineerde met de hoge resistentie van NoV ten opzichte van frequent gebruikte desinfectie praktijken (bv. wassen), dat NoV zullen overleven gedurende de tijdsperiode tussen contaminatie en consumptie. Dit illustreert het belang van een preventieve aanpak als strategie ter beheersing van NoV in de G&F productieketen.

In **Hoofdstuk 2** en **Hoofdstuk 3** is het arsenaal van in ons labo beschikbare methoden voor de detectie van NoV in de G&F productieketen aangevuld met de selectie van een methode voor de detectie in irrigatie- en waswater en met een methode voor detectie van NoV op oppervlakken. Zo werden in **Hoofdstuk 2** vier virale concentratiemethoden geëvalueerd voor de recovery van MNV-1 en MS2 in waswater (1 L) en vier types irrigatie water (grondwater, regenwater, open-put water en rivierwater) (2-5 L). De beste optie bleek ‘Methode 1’ die gebaseerd is op het virus adsorptie en elutie (VIRADEL) principe en gebruik maakt van een elektronegatieve HA-filter in combinatie met een basische elutie-buffer op basis van Triton X-100 en een secondaire concentratie methode gebaseerd op precipitatie met behulp van polyethyleenglycol (PEG). Methode 1 werd eveneens

geëvalueerd voor de detectie van humane voedsel-gebonden virussen zoals HAV, GI en GII NoV, en RV in dezelfde vijf verschillende watertypes als eerder aangehaald. Tot slot werd aangetoond dat Methode 1 een betrouwbare methode is voor de detectie van HAV, GI en GII NoV. Maar voor detectie van RV werd deze methode als minder efficiënt ervaren aangezien een significant lagere recovery efficiëntie (RE) bekomen werd voor RV in alle vijf de geteste water matrices (RE <2%).

In **Hoofdstuk 3** zijn drie verschillende swab elutie/extractie strategieën met elkaar vergeleken, waaronder één strategie gebaseerd op het recent verschenen ISO protocol (ISO/TS 15216-1),om het swab protocol te optimaliseren voor de detectie van virussen op (voedsel) contactoppervlakken. Zo werd een verbetering voorgesteld van het huidige ISO/TS protocol voor de detectie van NoV en HAV op oppervlakken aangezien de recovery efficiënties (RE) significant hoger waren met de nieuw voorgestelde semi-directe lyse strategie (strategie 3). Tevens werd het gebruik van katoenen swabs, polyester swabs en biowipes (Biomérieux) vergeleken voor de detectie van NoV GI en GII op de oppervlakken HD-PE, neopreen rubber en nitril handschoenen. Biowipes werden geïdentificeerd als de beste optie voor detectie van NoV op het ruwere neopreen rubber, echter geen significant verschil werd opgemerkt in RE bekomen voor de andere twee test oppervlakken.

In **Hoofdstuk 4** werden 14 loten bevroren frambozen, bedoeld voor de productie van puree, bemonsterd voor de aanwezigheid van NoV (GI & GII). Deze actie werd herhaald voor 12 loten IQF frambozen. Een bemonsteringsplan werd toegepast (n=5, c=0) zoals momenteel wordt uitgevoerd ter controle van ingevoerde bevroren aardbeien uit China. Een beslissingsmodule werd ontwikkeld om interpretatie van de NoV RT-qPCR resultaten te structureren. De toegevoegde waarde werd aangetoond van een bijkomende bevestigingsstap van positieve RT-qPCR signalen (door middel van sequeneren van het amplicon) daar vals-positieve monsters (hoewel alle negatieve controles negatief waren) op deze manier werden geïdentificeerd. Deze studie toonde tevens de aanwezigheid aan van kwantificeerbare hoeveelheden NoV, geclusterd in verschillende positieve monsters in één lot, wat duidt op sanitaire problemen. Verder werden de limitaties van monstername op lot niveau en van de huidige detectie methoden voor NoV in G&F bediscussieerd. Zo blijft het de vraag of monstername op lot niveau kosteneffectief is en de beste manier is om voedselveiligheid te garanderen aangezien bemonstering de afwezigheid van NoV positieve units in een lot niet kan garanderen. De benodigde aantal monsters voor de detectie van lage aantallen, met een lage frequentie en niet-uniform verdeelde contaminatie is namelijk onpraktisch groot. Voorts werd het standaard toevoegen van een proces controle (PC) en het standaard rapporteren van resulterende data aangemoedigd, hoewel

verder onderzoek vereist is voor het verbeteren van de beschikbaarheid van betrouwbare controles.

In **Hoofdstuk 5** en **Hoofdstuk 6** is dieper ingegaan op de potentiële rol van was- en irrigatiewater als bron van contaminatie met voedsel-gebonden virussen in de G&F productieketen. In **Hoofdstuk 5** werd kritisch inzicht verkregen op waswater als potentiële bron voor kruiscontaminatie. In dit hoofdstuk werd kwantitatieve data verzameld, gebruik makend van een dynamisch twee-stap was proces (geen desinfectantia werden gebruikt) als simulatie voor een commercieel sla was proces. Twee processen van kruiscontaminatie (sla naar water en water naar sla) werden bekeken in de simulatiestudie en de overdracht van *E. coli*, *E. coli* O157, MS2 faag, en MNV-1 werd gekwantificeerd. De overdracht van deze micro-organismen van water naar sla werd bepaald door contaminatie van wasbad 1 (WB1) met drie verschillend inoculatie niveaus. Naast kruiscontaminatie tussen de beide wasbaden werd tevens de initieel niet-gecontamineerde sla gecontamineerd door contact met water in WB1. Transfer ratios, welke de kruiscontaminatie van micro-organismen van geïnoculeerd WB1 naar de sla kwantificeren, varieerden tussen 0.3% tot 1.5%. De data bekomen in deze studie benadrukt de kwetsbaarheid van G&F voor kruiscontaminatie en illustreert de noodzaak voor het gebruik van gepaste desinfectantia om kruiscontaminatie te voorkomen.

In **Hoofdstuk 6** worden de beschikbare QMRA studies besproken die water includeren als transmissieroute van contaminatie en/of als desinfectie strategie, in de productieketen van G&F. Het meest frequent geïdentificeerde ontbrekende gegeven was de beschikbaarheid van plaats-specifieke data. Meer bepaald data over mogelijke internalisatie en overleving van de pathogenen in gewassen vertoonden gegevenstekorten. Net als de overdracht van pathogenen in irrigatie water en andere waterbronnen (bv. waswater), naar de gewassen. Dit laatste illustreert de relevantie van het onderzoek in Hoofdstuk 5.

De kennis verworven in bovenstaande hoofdstukken heeft geleid tot het formuleren van algemene conclusies in **Hoofdstuk 7**. Men wordt geconfronteerd met allerlei onzekerheden wanneer men de problematiek van NoV in de productieketen van G&F probeert te doorgronden. In Hoofdstuk 7 werd dieper ingegaan op deze onzekerheden gerelateerd aan de inschatting van het gezondheidsrisico, gestandaardiseerde detectie, prevalentie en testen, en de relevantie van indicatoren en controle opties. Eveneens werden perspectieven voor verder onderzoek aangebracht.

REFERENCE LIST

REFERENCE LIST

Agudo, A., Slimaani, N., and Ocke, M. C. (2002) Consumption of vegetables, fruit and other plant foods in the European prospective investigation into cancer and nutrition (EPIC) cohorts from 10 European countries. *Public Health Nutrition* **5**, 1179-1196.

Agullo-Barcelo, M., Oliva, F., and Lucena, F. (2013) Alternative indicators for monitoring *Cryptosporidium* oocysts in reclaimed water. *Environmental Science and Pollution Research* **20**, 4448-4454.

Ahmed, S.M., Lopman, B.A. and Levy, K. (2013) A systematic review and meta-analysis of the global seasonality of norovirus. *Plos One* **8**.

Ahmed,W., Richardson,K., Sidhu,J.P.S., Jagals,P., and Toze,S. (2014) Inactivation of faecal indicator bacteria in a roof-captured rainwater system under ambient meteorological conditions. *Journal of Applied Microbiology* **116**, 199-207.

Aiello,R., Cirelli,G.L., Consoli,S., Licciardello,F., and Toscano,A. (2013) Risk assessment of treated municipal wastewater reuse in Sicily. *Water Science and Technology* **67**, 89-98.

Akhter, J., Alhajjar, S., Myint, S. and Qadri, S.M.H. (1995) Viral contamination of environmental surfaces on a general pediatric ward and playroom in a major referral center in Riyadh. *European Journal of Epidemiology* **11**, 587-590.

Al-Juaidi,A.E., Kaluarachchi,J.J., and Kim,U. (2010) Multi-criteria decision analysis of treated wastewater use for agriculture in water deficit regions. *Journal of the American Water Resources Association* **46**, 395-411.

Albinana-Gimenez, N., Clemente-Casares, P., Cagua, B., Huguet, J.M., Courtois, S. and Girones, R. (2009) Comparison of methods for concentrating human adenoviruses, polyomavirus JC and noroviruses in source waters and drinking water using quantitative PCR. *Journal of Virological Methods* **158**, 104-109.

Alexander, W.J., Holmes, J.R., Shaw, J.F.E., Riley, W.E. and Roper, W.L. (1986) Norwalk virus outbreak at a college campus. *Southern Medical Journal* **79**, 33-36.

Allende, A., Aguayo, E. and Artés, F. (2004) Microbial and sensory quality of commercial fresh processed red lettuce throughout the production chain and shelf life. *International Journal of Food Microbiology* **91**, 109-117.

Allende, A., Selma, M.V., Lopez-Galvez, F., Villaescusa, R. and Gil, M.I. (2008) Impact of wash water quality on sensory and microbial quality, including *Escherichia coli* cross-contamination, of fresh-cut escarole. *Journal of Food Protection* **71**, 2514-2518.

Allwood, P.B., Malik, Y.S., Hedberg, C.W. and Goyal, S.M. (2004) Effect of temperature and sanitizers on the survival of feline calicivirus, Escherichia coli, and F-specific coliphage MS2 on leafy salad vegetables. *Journal of Food Protection* **67**, 1451-1456.

Alum, A., Enriquez, C. and Gerba, C.P. (2011) Impact of drip irrigation method, soil, and virus type on tomato and cucumber contamination. *Food and Environmental Virology* **3**, 78-85.

Amoah, P., Drechsel, P., Abaidoo, R.C., and Klutse, A. (2007a) Effectiveness of common and improved sanitary washing methods in selected cities of West Africa for the reduction of coliform bacteria and helminth eggs on vegetables. *Tropical medicine & international health* **12**, 40-50.

Amoah, P., Dreschel, P., Henseler, M., and Abaidoo, R. C. (2007b) Irrigated urban vegetable production in Ghana: microbiological contamination in farms and markets and associated consumer risk groups. *Journal of Water and Health* **5**, 455-466.

Amoah, P., Keraita, B., Akple, M., Drechsel, P., Abaidoo, R.C., and Konradsen, F. (2011) Low-cost options for reducing consumer health risks from farm to fork where crops are irrigated with polluted water in West Africa. Colombo, Sri Lanka: International Water Management Institute. 45p. (IWMI Research Report 141). Doi: 10.5337/2011.201. Available online: http://www.iwmi.cgiar.org/Publications/IWMI_Research_Reports/PDF/PUB141/RR141.pdf

An, W., Zhang, D. Q., Xiao, S. M., Yu, J. W., and Yang, M. (2011) Quantitative health risk assessment of *Cryptosporidium* in rivers of southern China based on continous monitoring. *Environmental Science & Technology* **45**, 4951-4958.

Anderson, A.D., Garrett, V.D., Sobel, J., Monroe, S.S., Fankhauser, R.L., Schwab, K.J., Bresee, J.S., Mead, P.S., Higgins, C., Campana, J., Glass, R.I. and Outbreak Invest, T. (2001) Multistate outbreak of Norwalk-like virus gastroenteritis associated with a common caterer. *American Journal of Epidemiology* **154**, 1013-1019.

Anderson, S.A., Turner, S.J. and Lewis, G.D. (1997) Enterococci in the New Zealand environment: implications for water quality monitoring. *Water Science and Technology* **35**, 325-331.

Anonymous (1995) ISO 10705-1. Water quality, detection and enumeration of bacteriophages - Part 1: enumeration of F-specific RNA bacteriophages. Geneva: International Organisation for Standardisation.

Anonymous (2000) ISO 9308-1:2000. Water quality - detection and enumeration of *Escherichia coli* and coliform bacteria - Part 1: Membrane filtration method. Geneva: International Organisation for Standardisation.

Anonymous (2002) ISO 15705:2002. Water quality - Determination of the chemical oxygen demand index (ST-COD) - Small-scale sealed-tube method. Geneva: International Organisation for Standardisation.

Anonymous (2004) ISO 18593. Microbiology of food and animal feeding stuffs - Horizontal methods for sampling techniques from surfaces using contact plates and swabs.

Anonymous (2005) Commission regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Official Journal of the European Communities (7/12/2007) (inclusive EU Regulation 1441/2007).

Anonymous (2012a) Commission Implementing Regulation (EU) 1235/2012 of 19 December 2012 amending Annex I to Regulation 669/2009 implementing Regulation 882/2004 of the European Parliament and of the Council as regards the increased level of official controls on imports of certain food and feed of non-animal origin. *Official Journal of the European Union 20/12/12*, L350/344.

Anonymous (2012b) Decree (executive order No. 970 of 28/09/2012) amending the decree on foodstuffs concerning heat treatment of frozen raspberries: Danish food administration. Available online: https://www.retsinformation.dk/Forms/R0710.aspx?id=143397 (October 2014).

Anonymous (2012c) ISO/TS 15216-1. Microbiology of food and animal feed-Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR-Part 1: Method for quantification.

Anonymous (2012d) Tenazität (widerstandsfähigkeit) von noroviren in erdbeerkompott. *Stellungnahme Nr038/2012 des Bundesinstitut f • r Riskobewertung (BfR)http://wwwbfrbundde/de/a-z_index/noroviren-5090html*.

Anonymous (2013) ISO/DIS 16140-1. Microbiology of food and animal feed - Method validation - Part 1: Vocabulary.

Ansari, S.A., Sattar, S.A., Springthorpe, V.S., Wells, G.A. and Tostowaryk, W. (1989) In vivo protocol for testing efficacy of hand-washing agents against viruses and bacteria: experiments with rotavirus and escherichia coli. *Applied and Environmental Microbiology* **55**, 3113-3118.

Apha (1998) Standard methods for the examination of water and wastewater. pp.1-1220. Washington, D.C.: American Public Health Association.

Artes, F. and Allende, A. (2005) Processing lines and alternative preservation techniques to prolong the shelf-life of minimally fresh processed leafy vegetables. *European Journal of Horticultural Science* **70**, 231-245.

Artes, F., Gomez, P., Aguayo, E., Escalona, V. and Artes-Hernandez, F. (2009) Sustainable sanitation techniques for keeping quality and safety of fresh-cut plant commodities. *Postharvest Biology and Technology* **51**, 287-296.

Arthur, L., Jones, S., Fabri, M. and Odumeruz, J. (2007) Microbial survey of selected Ontario-grown fresh fruits and vegetables. *Journal of Food Protection* **70**, 2864-2877.

Asano,T., Leong,L.Y.C., Rigby,M.G., and Sakaji,R.H. (1992) Evaluation of the California waste-water reclamation criteria using enteric virus monitoring data. *Water Science and Technology* **26**, 1513-1524.

Atmar, R.L. (2010) Noroviruses: State of the art. *Food and Environmental Virology* **2**, 117-126.

Atmar, R.L. and Estes, M.K. (2006) The epidemiologic and clinical impartance of norovirus infection. *Gastroenterology Clinics of North America* **35**, 275-290.

Atmar, R.L., Opekun, A.R., Gilger, M.A., Estes, M.K., Crawford, S.E., Neill, F.H. and Graham, D.Y. (2008) Norwalk virus shedding after experimental human infection. *Emerging Infectious Diseases* **14**, 1553-1557.

Aycicek, H., Oguz, U. and Karci, K. (2006) Determination of total aerobic and indicator bacteria on some raw eaten vegetables from wholesalers in Ankara, Turkey. *International Journal of Hygiene and Environmental Health* **209**, 197-201.

Ayuso-Gabella,N., Page,D., Masciopinto,C., Aharoni,A., Salgot,M., and Wintgens,T. (2011) Quantifying the effect of Managed Aquifer Recharge on the microbiological human health risks of irrigating crops with recycled water. *Agricultural Water Management* **99**, 93-102.

Azizkhani, M., Elizaquivel, P., Sanchez, G., Selma, M.V. and Aznar, R. (2013) Comparative efficacy of Zataria multiflora Boiss., Origanum compactum and Eugenia caryophyllus essential oils against *E. coli* O157:H7, feline calicivirus and endogenous microbiota in commercial baby-leaf salads. *International Journal of Food Microbiology* **166**, 249-255.

Bae, J. and Schwab, K.J. (2008) Evaluation of murine norovirus, feline calicivirus, poliovirus, and MS2 as surrogates for human norovirus in a model of viral persistence in surface water and groundwater. *Applied and Environmental Microbiology* **74**, 477-484.

Baert, L. (2009) Molecular detection of, and strategies to reduce Norovirus load or infectivity in foods: PhD dissertation, Faculty of Bioscience Engineering, Ghent University, Ghent.

Baert, L., Debevere, J. and Uyttendaele, M. (2009a) The efficacy of preservation methods to inactivate foodborne viruses. *International Journal of Food Microbiology* **131**, 83-94.

Baert, L., Mattison, K., Loisy-Hamon, F., Harlow, J., Martyres, A., Lebeau, B., Stals, A., Van Coillie, E., Herman, L. and Uyttendaele, M. (2011) Review: Norovirus prevalence in Belgian, Canadian and French fresh produce: A threat to human health? *International Journal of Food Microbiology* **151**, 261-269.

Baert, L., Uyttendaele, M. and Debevere, J. (2007) Evaluation of two viral extraction methods for the detection of human noroviruses in shellfish with conventional and real-time reverse transcriptase PCR. *Letters in Applied Microbiology* **44**, 106-111.

Baert, L., Uyttendaele, M. and Debevere, J. (2008a) Evaluation of viral extraction methods on a broad range of Ready-To-Eat foods with conventional and real-time RT-PCR for Norovirus GII detection. *International Journal of Food Microbiology* **123**, 101-108.

Baert, L., Uyttendaele, M., Van Coillie, E. and Debevere, J. (2008b) The reduction of murine norovirus 1, B-fragilis HSP40 infecting phage B40-8 and *E-coli* after a mild thermal pasteurization process of raspberry puree. *Food Microbiology* **25**, 871-874.

Baert, L., Uyttendaele, M., Vermeersch, M., Van Coillie, E. and Debeverei, J. (2008c) Survival and transfer of murine norovirus 1, a surrogate for human noroviruses, during the production process of deep-frozen onions and spinach. *Journal of Food Protection* **71**, 1590-1597.

Baert, L., Vandekinderen, I., Devlieghere, F., Van Coillie, E., Debevere, J. and Uyttendaele, M. (2009b) Efficacy of sodium hypochlorite and peroxyacetic acid to reduce murine norovirus 1, B40-8, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 on shredded iceberg lettuce and in residual wash water. *Journal of Food Protection* **72**, 1047-1054.

Baert, L., Wobus, C.E., Van Coillie, E., Thackray, L.B., Debevere, J. and Uyttendaele, M. (2008d) Detection of murine norovirus 1 by using plaque assay, transfection assay, and real-time reverse transcription-PCR before and after heat exposure. *Applied and Environmental Microbiology* **74**, 543-546.

Barker,S.F., O'Toole,J., Sinclair,M.I., Leder,K., Malawaraarachchi,M., and Hamilton,A.J. (2013) A probabilistic model of norovirus disease burden associated with greywater irrigation of home-produced lettuce in Melbourne, Australia. *Water Research* **47**, 1421-1432.

Barker-Reid, F., Harper, G. A., and Hamilton, A. J. (2010) Affluent effluent: growing vegetables with wastewater in Melbourne, Australia - a wealthy but bone-dry city. *Irrigation and Drainage Systems* **24**, 79-94.

Barrabeig, I., Rovira, A., Buesa, J., Bartolome, R., Pinto, R., Prellezo, H. and Dominguez, A. (2010) Foodborne norovirus outbreak: the role of an asymptomatic food handler. *Bmc Infectious Diseases* **10**, 7 pp.

Bassett, J. and McClure, P. (2008) A risk assessment approach for fresh fruits. *Journal of Applied Microbiology* **104**, 925-943.

Bastos, R.K.X., Bevilacqua, P.D., Silva, C.A.B. and Silva, C.V. (2008) Wastewater irrigation of salad crops: further evidence for the evaluation of the WHO guidelines. *Water Science and Technology* **57**, 1213-1219.

Battistone, A., Buttinelli, G., Bonomo, P., Fiore, S., Amato, C., Mercurio, P., Cicala, A., Simeoni, J., Foppa, A., Triassi, M., Pennino, F. and Fiore, L. (2014) Detection of enteroviruses in influent and effluent flow samples from wastewater treatment plants in Italy. *Food and Environmental Virology* **6**, 13-22.

Baur, S., Klaiber, R., Hammes, W.P. and Carle, R. (2004) Sensory and microbiological quality of shredded, packaged iceberg lettuce as affected by pre-washing procedures with chlorinated and ozonated water. *Innovative Food Science & Emerging Technologies* **5**, 45-55.

Baur, S., Klaiber, R., Wei, H., Hammes, W.P. and Carle, R. (2005) Effect of temperature and chlorination of pre-washing water on shelf-life and physiological properties of ready-to-use iceberg lettuce. *Innovative Food Science & Emerging Technologies* **6**, 171-182.

Belliot, G., Lavaux, A., Souihel, D., Agnello, D. and Pothier, P. (2008) Use of murine norovirus as a surrogate to evaluate resistance of human norovirus to disinfectants. *Applied and Environmental Microbiology* **74**, 3315-3318.

Belliot, G., Loutreul, J., Estienney, M., Cazeaux, C., Nicorescu, I., Aho, S., Gervais, P., Orange, N., Pothier, P. and Morin, T. (2013) Potential of pulsed light to inactivate bacteriophage MS2 in simple liquid medium and on complex foodstuffs. *Food and Environmental Virology* **5**, 176-179.

Bellou, M., Kokkinos, P. and Vantarakis, A. (2013) Shellfish-borne viral outbreaks: A systematic review. *Food and Environmental Virology* **5**, 13-23.

Benabbes, L., Ollivier, J., Schaeffer, J., Parnaudeau, S., Rhaissi, H., Nourlil, J. and Le Guyader, F.S. (2013) Norovirus and other human enteric viruses in Moroccan shellfish. *Food and Environmental Virology* **5**, 35-40.

Bernard, H., Faber, M., Wilking, H., Haller, S., H¨hle, M., Schielke, A., Ducomble, T., Siffczyk, C., Merbercks, S.S., Fricke, G., Hamouda, O., Stark, K., Werber, D. and Team, o.b.o.t.O.I. (2014) Large multistate outbreak of norovirus gastroenteritis associated with frozen strawberries, Germany, 2012. *Euro Surveillance* **19**, 9 pp.

Bertrand, I., Schijven, J.F., Sanchez, G., Wyn-Jones, P., Ottoson, J., Morin, T., Muscillo, M., Verani, M., Nasser, A., Husman, A.M.D., Myrmel, M., Sellwood, J., Cook, N. and Gantzer, C. (2012) The impact of temperature on the inactivation of enteric viruses in food and water: a review. *Journal of Applied Microbiology* **112**, 1059-1074.

Beuchat, L.R. (1998) Surface decontamination of fruits and vegetables eaten raw: a review. *World Health Organisation, Food Safety Issues*, WHO/FSF/FOS/98.92. Available on line: www.who.int/foodsafety/publications/fs_management/en/surface_decon.pdf.

BfR (2013) Sicher verpflegt. Besonders empfindliche personengruppen in Gemeinschaftseinrichtungen. [Safe care. Particluarly vulnerable groups in community facilities]. *Bundesinstitut für Risikobewertung, Berlin, Germany*.

Bhagwat, A.A. (2006) Microbiological safety of fresh-cut produce: where are we now? In *Microbiology of fresh produce* ed. Matthews, K.R. Washington DC: ASM Press.

Bidawid, S., Farber, J.M. and Sattar, S.A. (2000) Inactivation of hepatitis A virus (HAV) in fruits and vegetables by gamma irradiation. *International Journal of Food Microbiology* **57**, 91-97.

Bidawid, S., Farber, J.M. and Sattar, S.A. (2001) Survival of hepatitis A virus on modified atmosphere-packaged (MAP) lettuce. *Food Microbiology* **18**, 95-102.

Bidawid, S., Malik, N., Adegbunrin, O., Sattar, S.A. and Farber, J.M. (2004) Norovirus cross-contamination during food handling and interruption of virus transfer by hand antisepsis: Experiments with feline calicivirus as a surrogate. *Journal of Food Protection* **67**, 103-109.

Blaise-Boisseau, S., Hennechart-Collette, C., Guillier, L. and Perelle, S. (2010) Duplex real-time qRT-PCR for the detection of hepatitis A virus in water and raspberries using the MS2 bacteriophage as a process control. *Journal of Virological Methods* **166**, 48-53.

Blumenthal, U. and Peasey, A. (2002) Critical review of epidemiological evidence of the health effects of wastewater and excreta use in agriculture. Unpublished document prepared for WHO (available upon request), World Health Organization, Geneva.

Bofill-Mas, S., Albinana-Gimenez, N., Clemente-Casares, P., Hundesa, A., Rodriguez-Manzano, J., Allard, A., Calvo, M. and Girones, R. (2006) Quantification and stability of human adenoviruses and polyomavirus JCPyV in wastewater matrices. *Applied and Environmental Microbiology* **72**, 7894-7896.

Bolton, S.L., Kotwal, G., Harrison, M.A., Law, S.E., Harrison, J.A. and Cannon, J.L. (2013) Sanitizer efficacy against murine norovirus, a surrogate for human norovirus, on stainless steel surfaces when using three application methods. *Applied and Environmental Microbiology* **79**, 1368-1377.

Boone, S.A. and Gerba, C.P. (2007) Significance of fomites in the spread of respiratory and enteric viral disease. *Applied and Environmental Microbiology* **73**, 1687-1696.

Boraychuk, V.M., Bradbury, R.W., Dimock, R., Fehr, M., Gensler, G.E., King, R.K., Rieve, R. and Barrios, P.R. (2009) A microbiological survey of selected Alberta-grown fresh produce from farmers' markets in Alberta, Canada. *Journal of Food Protection* **72**, 415-420.

Borchardt, M.A., Haas, N.L. and Hunt, R.J. (2004) Vulnerability of drinking-water wells in La Crosse, Wisconsin, to enteric-virus contamination from surface water contributions. *Applied and Environmental Microbiology* **70**, 5937-5946.

Bosch, A., Bidawid, S., Le Guyader, S., Lees, D. and Jaykus, L.A. (2011) Norovirus and hepatitis A virus in shellfish, soft fruits and water. In *Rapid detection, identification, and quantification of foodborne pathogens* ed. Hoorfar, J.: 2011 ASM Press.

Bosch, A., Pinto, R.M. and Abad, F.X. (2006) Survival and transport of enteric viruses in the environment. In *Viruses in foods* ed. Goyal, S.M. p.345. United States of America: Springer

Bowen, A., Fry, A., Richards, G. and Beuchat, L. (2006) Infections associated with cantaloupe consumption: a public health concern. *Epidemiology and Infection* **134**, 675-685.

Boxman, I.L.A., Dijkman, R., Loeke, N.A.J.M., Hagele, G., Tilburg, J.J.H.C., Vennema, H. and Koopmans, M. (2009) Environmental swabs as a tool in norovirus outbreak investigation, including outbreaks on cruise ships. *Journal of Food Protection* **72**, 111-119.

Boxman, I.L.A., Verhoef, L., Dijkman, R., Hagele, G., Loeke, N.A.J.M. and Koopmans, M. (2011) Year-round prevalence of norovirus in the environment of catering companies without a recently reported outbreak of gastroenteritis. *Applied and Environmental Microbiology* **77**, 2968-2974.

Boyer, R.R. (2006) Mechanisms associated with attachment of *Escherichia coli* O157:H7 to lettuce surfaces. PhD thesis, Virginia Polytechnic Institute and State University, VA, USA.

Bozkurt, H., D'Souza, D.H. and Davidson, M. (2014a) Thermal inactivation of human norovirus surrogates in spinach and measurement of its uncertainty. *Journal of Food Protections* **77**, 276-283.

Bozkurt, H., D'Souza, D.H. and Davidson, P.M. (2014b) A comparison of the thermal inactivation kinetics of human norovirus surrogates and hepatitis A virus in buffered cell culture medium. *Food Microbiology* **42**, 212-217.

Bradford,S.A., Morales,V.L., Zhang,W., Harvey,R.W., Packman,A.I., Mohanram,A., and Welty,C. (2013) Transport and fate of microbial pathogens in agricultural settings. *Critical Reviews in Environmental Science and Technology* **43**, 775-893.

Brandao, M.L.L., Almeida, D.O., Bispo, F.C.P., Bricio, S.M.L., Marin, V.A. and Miagostovich, M.P. (2014) Assessment of microbiological contamination of fresh, minimally processed, and ready-to-eat lettuces (*Lactuca sativa*), Rio de Janeiro State, Brazil. *Journal of Food Science* **79**, M961-M966.

Brassard, J., Gagne, M.J., Genereux, M. and Cote, C. (2012) Detection of human food-borne and zoonotic viruses on irrigated, field-grown strawberries. *Applied and Environmental Microbiology* **78**, 3763-3766.

BRC, Water UK and ADAS (2001) *The safe sludge matrix. Guidelines for the application of sewage sludge to agricultural land*: Britisch Retail Consortium; Water UK; ADAS.

Buchholz, A.L., Davidson, G.R., Marks, B.P., Todd, E.C.D. and Ryser, E.T. (2014) Tracking an *Escherichia coli* O157:H7-contaminated batch of leafy greens through a pilot-scale fresh-cut processing line. *Journal of Food Protection* **77**, 1487-1494.

Buchholz, U., Bernard, H., Werber, D., Bohmer, M.M., Remschmidt, C., Wilking, H., Delere, Y., an der Heiden, M., Adlhoch, C., Dreesman, J., Ehlers, J., Ethelberg, S., Faber, M., Frank, C., Fricke, G., Greiner, M., Hohle, M., Ivarsson, S., Jark, U., Kirchner, M., Koch, J., Krause, G., Luber, P., Rosner, B., Stark, K. and Kuhne, M. (2011) German outbreak of *Escherichia coli* O104:H4 associated with sprouts. *New England Journal of Medicine* **365**, 1763-1770.

Bull, R.A., Tanaka, M.M. and White, P.A. (2007) Norovirus recombination. *Journal of General Virology* **88**, 3347-3359.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J. and Wittwer, C.T. (2009) The MIQE guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry* **55**, 611-622.

Butot, S., Putallaz, T., Amoroso, R. and Sanchez, G. (2009) Inactivation of enteric viruses in minimally processed berries and herbs. *Applied and Environmental Microbiology* **75**, 4155-4161.

Butot, S., Putallaz, T. and Sanchez, G. (2008) Effects of sanitation, freezing and frozen storage on enteric viruses in berries and herbs. *International Journal of Food Microbiology* **126**, 30-35.

Butot, S., Zuber, S. and Baert, L. (2014) Sample preparation prior to molecular amplification: complexities and opportunities. *Current Opinion in Virology* **4**, 66-70.

CAC (1999) CAC/GL 30-1999. Principles and guidelines for the conduct of MRA. Available online: http://www.fao.org/docrep/005/y1579e/y1579e05.htm

CAC (2007a) CAC/GL 63-2007. Principles and guidelines for the conduct of microbiological risk management (MRM). Available online: http://www.codexalimentarius.org/standards/list-of-standards/en/

CAC (2007b) CAC/GL 62-2007. Working principles for risk analysis for food safety for application by governments. Available online: http://www.codexalimentarius.org/standards/list-of-standards/en/

Calder, L., Simmons, G., Thornley, C., Taylor, P., Pritchard, K., Greening, G. and Bishop, J. (2003) An outbreak of hepatitis A associated with consumption of raw blueberries. *Epidemiology and Infection* **131**, 745-751.

Cann, K.F., Thomas, D.R., Salmon, R.L., Wyn-Jones, A.P. and Kay, D. (2013) Extreme water-related weather events and waterborne disease. *Epidemiology and Infection* **141**, 671-686.

Cannon, J.L., Papafragkou, E., Park, G.W., Osborne, J., Jaykus, L.A. and Vinje, J. (2006) Surrogates for the study of norovirus stability and inactivation in the environment: A comparison of murine norovirus and feline calicivirus. *Journal of Food Protection* **69**, 2761-2765.

Carducci, A., Battistini, R., Rovini, E. and Verani, M. (2009) Viral removal by wastewater treatment: monitoring of indicators and pathogens. *Food and Environmental Virology* **1**, 85-91.

Carducci, A., Verani, M., Casini, B., Giuntini, A., Mazzoni, F., Rovini, E., Passaglia, A., Giusti, L., Valenza, A. and Lombardi, R. (2002) Detection and potential indicators of the presence of hepatitis C virus on surfaces in hospital settings. *Letters in Applied Microbiology* **34**, 189-193.

Carducci, A., Verani, M., Lombardi, R., Casini, B. and Privitera, G. (2011) Environmental survey to assess viral contamination of air and surfaces in hospital settings. *Journal of Hospital Infection* **77**, 242-247.

Carrasco,E., Perez-Rodriguez,F., Valero,A., Garcia-Gimeno,R.M., and Zurera,G. (2010) Risk assessment and management of *Listeria Monocytogenes* in ready-to-eat lettuce salads. *Comprehensive Reviews in Food Science and Food Safety* **9**, 498-512.

Carratala, A., Rodriguez-Manzano, J., Hundesa, A., Rusinol, M., Fresno, S., Cook, N. and Girones, R. (2013a) Effect of temperature and sunlight on the stability of human adenoviruses and MS2 as fecal contaminants on fresh produce surfaces. *International Journal of Food Microbiology* **164**, 128-134.

Carratala,A., Rusinol,M., Rodriguez-Manzano,J., Guerrero-Latorre,L., Sommer,R., and Girones,R. (2013b) Environmental effectors on the inactivation of human adenoviruses in water. *Food and Environmental Virology* **5**, 203-214.

Carter, M.J. (2005) Enterically infecting viruses: pathogenicity, transmission and significance for food and waterborne infection. *Journal of Applied Microbiology* **98**, 1354-1380.

Cashdollar, J.L., Brinkman, N.E., Griffin, S.M., McMinn, B.R., Rhodes, E.R., Varughese, E.A., Grimm, A.C., Parshionikar, S.U., Wymer, L. and Fout, G.S. (2013) Development and Evaluation of EPA Method 1615 for Detection of Enterovirus and Norovirus in Water. *Applied and Environmental Microbiology* **79**, 215-223.

Cashdollar, J.L. and Wymer, L. (2013) Methods for primary concentration of viruses from water samples: a review and meta-analysis of recent studies. *Journal of Applied Microbiology* **115**, 1-11.

Casteel, M.J., Schmidt, C.E. and Sobsey, M.D. (2008) Chlorine disinfection of produce to inactivate hepatitis A virus and coliphage MS2. *International Journal of Food Microbiology* **125**, 267-273.

Casteel, M.J., Schmidt, C.E. and Sobsey, M.D. (2009) Chlorine inactivation of coliphage MS2 on strawberries by industrial-scale water washing units. *Journal of Water and Health* **7**, 244-250.

Castro-Rosas, J. and Escartin, E.F. (2000) Survival and growth of *Vibrio cholerae* 01, *Salmonella typhi*, and *Escherichia coli* O157 : H7 in alfalfa sprouts. *Journal of Food Science* **65**, 162-165.

Caul, E.O. (1994) Small round structured viruses – airborne transmission and hospital control. *Lancet* **343**, 1240-1242.

CDC (1996) Update: Outbreaks of *Cyclospora cayetanensis* infection-United States and Canada, 1996. *Morbidity and Mortality Weekly Report* **45**, 611-612.

CDC (2000) Appendix B. Guidelines for confirmation of foodborne-disease outbreak. *Morbidity and Mortality Weekly Report* **49**, 54-62.

CDC (2008) Summary of notifiable diseases- United States 2006. *Morbidity and Mortality Weekly Report* **55**, 1-94.

CDC (2009) Surveillance for foodborne disease outbreaks - United States, 2006. *MMWR Surveillance summaries* **58**, 609-615.

CDC (2010) Investigation update: Multistate outbreak of human *E. coli* O145 infections linked to shredded Romaine lettuce from a single processing facility. *Centers for Disease Control and Prevention*.

CDC (2013) Multistate outbreak of hepatitis A virus infections linked to pomegranate seeds from Turkey (Final Update). *Centers for Disease Control and Prevention*, Accessed September 2014. http://www.cdc.gov/hepatitis/Outbreaks/2013/A2011b-2003-2031/index.html.

CDC (2014) Technical fact sheet: Burden of norovirus illness and outbreaks. *http://wwwcdcgov/norovirus/php/illness-outbreakshtml (last accessed on 15th August 2014)*.

Cefas (2013) European Union Reference Laboratory (EURL) proficiency testing scheme. Norovirus (Genogroup I and II) and hepatitis A virus proficiency testing 2013. Reference number PT 46. *Available online: https://eurlcefasorg/media/10543/proficiency-testing-46-virus-2012-final-reportpdf*, 1-22.

Cefas (2014) UK NRL meeting notes: EU Reference laboratory network for monitoring bacteriological and viral contamination of bivalve molluscs. Annual workshop, 13-15 May 2014. 13 pp.

Ceuppens, S., Rajkovic, A., Heyndrickx, M., Tsilia, V., van De Wiele, T., Boon, N., Uyttendaele, M. (2013) Regulation of toxin production by *Bacillus cereus* and its food safety implications. *Critical Reviews in Microbiology* **37**, 188-213.

Charles, K.J., Shore, J., Sellwood, J., Laverick, M., Hart, A. and Pedley, S. (2009) Assessment of the stability of human viruses and coliphage in groundwater by PCR and infectivity methods. *Journal of Applied Microbiology* **106**, 1827-1837.

Cheesbrough, J.S., Green, J., Gallimore, C.I., Wright, P.A. and Brown, D.W.G. (2000) Widespread environmental contamination with Norwalk-like viruses (NLV) detected in a prolonged hotel outbreak of gastroenteritis. *Epidemiology and Infection* **125**, 93-98.

Chen, H.Q., Hoover, D.G. and Kingsley, D.H. (2005) Temperature and treatment time influence high hydrostatic pressure inactivation of feline calicivirus, a norovirus surrogate. *Journal of Food Protection* **68**, 2389-2394.

Chen, Y., Dennis, S.B., Hartnett, E., Paoli, G., Pouillot, R., Ruthman, T. and Wilson, M. (2013) FDA-iRISK - A comparative risk assessment system for evaluating and ranking food-hazard pairs: Case studies on microbial hazards. *Journal of Food Protection* **76**, 376-385.

Cheong, S., Lee, C., Song, S.W., Choi, W.C., Lee, C.H. and Kim, S.J. (2009) Enteric viruses in raw vegetables and groundwater used for irrigation in South Korea. *Applied and Environmental Microbiology* **75**, 7745-7751.

Chiapponi, C., Pavoni, E., Bertasi, B., Baioni, L., Chiesa, E., Cianti, L., Losio, M.N. and Pongolini, S. (2014) Isolation and genomic sequence of hepatitis A virus from mixed frozen berries in Italy. *Food and Environmental Virology* **6**, 202-206.

Cho, H. G., Lee, S. G., Kim, W. H., Lee, J. S., Park, P. H., Cheon, D. S., Jheong, W. H., Jho, E. H., Lee, J. B. and Paik, S. Y. (2014) Short report: Acute gastroenteritis outbreaks associated with ground-waterborne norovirus in South Korea during 2008-2012. *Epidemiology & Infection* **142**, 2604-2609.

Choi, C., Song, I., Stine, S., Pimentel, J. and Gerba, C. (2004) Role of irrigation and wastewater reuse: comparison of subsurface irrigation and furrow irrigation. *Water Science and Technology* **50**, 61-68.

Colford, Jr., Roy, J. M., Beach, M. J., Hightower, A., Shaw, S. E., and Wade, T. J. (2006) A review of household drinking water intervention trials and an approach to the estimation of endemic waterborne gastroenteritis in the United States. *Journal of Water and Health* **4**, 71-88.

Costafreda, M.I., Bosch, A. and Pinto, R.M. (2006) Development, evaluation, and standardization of a real-time TaqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples. *Applied and Environmental Microbiology* **72**, 3846-3855.

Costan-Longares, A., Moce-Llivina, L., Avellon, A., Jofre, J. and Lucena, F. (2008) Occurrence and distribution of culturable enteroviruses in wastewater and surface waters of north-eastern Spain. *Journal of Applied Microbiology* **105**, 1945-1955.

Cotterelle, B., Drougard, C., Rolland, J., Becamel, M., Boudon, M., Pinede, S., Traor,, O., Balay, K.P.P. and Espi,, E. (2005) Outbreak of norovirus infection associated with the consumption of frozen raspberries, France, March 2005. *Eurosurveillance* **10**.

Coudray, C., Merle, G., Martin-Latil, S., Guillier, L. and Perelle, S. (2013) Comparison of two extraction methods for the detection of hepatitis A virus in lettuces using the murine norovirus as a process control. *Journal of Virological Methods* **193**, 96-102.

Croci, L., de Medici, D., Scalfaro, C., Fiore, A. and Toti, L. (2002) The survival of hepatitis A virus in fresh produce. *International Journal of Food Microbiology* **73**, 29-34.

Croci, L., Dubois, E., Cook, N., de Medici, D., Schultz, A.C., China, B., Rutjes, S.A., Hoorfar, J. and Van der Poel, W.H.M. (2008) Current methods for extraction and concentration of enteric viruses from fresh fruit and vegetables: Towards International Standards. *Food Analytical Methods* **1**, 73-84.

Cromeans, T., Park, G.W., Costantini, V., Lee, D., Wang, Q., Farkas, T., Lee, A. and Vinjé, J. (2014) Comprehensive comparison of cultivable norovirus surrogates in response to different inactivation and disinfection treatments. *Applied and Environmental Microbiology* **80**, 5743-5751.

Cubitt, W.D., Green, K.Y. and Payment, P. (1998) Prevalence of antibodies to the Hawaii strain of human calicivirus as measured by a recombinant protein based immunoassay. *Journal of Medical Virology* **54**, 135-139.

Cutolo, S.A., Piveli, R.P., Santos, J.G., Montes, C.R., Sundefeld, G., Campos, F., Gomes, T.M., and Melfi, A.J. (2012) Parasitological risk assessment from wastewater reuse for disposal in soil in developing countries. *Water Science and Technology* **65**, 1357-1367.

D'Agostino, M., Cook, N., Rodriguez-Lazaro, D. and Rutjes, S. (2011) Nucleic acid amplification-based methods for detection of enteric viruses: Definition of controls and interpretation of results. *Food and Environmental Virology* **3**, 55-60.

D'Souza, D.H., Sair, A., Williams, K., Papafragkou, E., Jean, J., Moore, C. and Jaykus, L. (2006) Persistence of caliciviruses on environmental surfaces and their transfer to food. *International Journal of Food Microbiology* **108**, 84-91.

da Silva, A.K., Le Saux, J.C., Parnaudeau, S., Pommepuy, M., Elimelech, M. and Le Guyader, F.S. (2007) Evaluation of removal of noroviruses during wastewater treatment, using real-time reverse transcription-PCR: Different behaviors of genogroups I and II. *Applied and Environmental Microbiology* **73**, 7891-7897.

Daelman, J., Jacxsens, L., Lahou, E., Devlieghere, F. and Uyttendaele, M. (2013) Assessment of the microbial safety and quality of cooked chilled foods and their production process. *International Journal of Food Microbiology* **160**, 193-200.

Damgaardlarsen, S., Jensen, K.O., Lund, E. and Nissen, B. (1977) Survival and movement of enterovirus in connection with land disposal of sludges. *Water Research* **11**, 503-508.

Dancer, D., Rangdale, R.E., Lowther, J.A. and Lees, D.N. (2010) Human norovirus RNA persists in seawater under simulated winter conditions but does not bioaccumulate efficiently in Pacific oysters (*Crassostrea gigas*). *Journal of Food Protection* **73**, 2123-2127.

Dancho, B.A., Chen, H.Q. and Kingsley, D.H. (2012) Discrimination between infectious and non-infectious human norovirus using porcine gastric mucin. *International Journal of Food Microbiology* **155**, 222-226.

Daniels, N.A., Bergmire-Sweat, D.A., Schwab, K.J., Hendricks, K.A., Reddy, S., Rowe, S.H., Fankhauser, R.L., Monroe, S.S., Atmar, R.L., Glass, R.I. and Mead, P. (2000) A foodborne outbreak of gastroenteritis associated with Norwalk-like viruses: First molecular traceback to deli sandwiches contaminated during preparation. *Journal of Infectious Diseases* **181**, 1467-1470.

Danyluk, M.D. and Schaffner, D.W. (2011) Quantitative assessment of the microbial risk of leafy greens from farm to consumption: Preliminary framework, data, and risk estimates. *Journal of Food Protection* **74**, 700-708.

Dawson, D.J., Paish, A., Staffell, L.M., Seymour, I.J. and Appleton, H. (2005) Survival of viruses on fresh produce, using MS2 as a surrogate for norovirus. *Journal of Applied Microbiology* **98**, 203-209.

De Boevre, M., Jacxsens, L., Lachat, C., Eeckhout, M., Di Mavungu, J. D., Audenaert, K., Haesaert, G., Kolsteren, P., De Meulenaer, B., and De Saeger, S. (2013) Human exposure to mycotoxins and their masked forms through cereal-based foods in Belgium. *Toxicology Letters* **218**, 281-292.

De Giusti, M., Aurigemma, C., Marinelli, L., Tufi, D., De Medici, D., Di Pasquale, S., De Vito, C. and Boccia, A. (2010) The evaluation of the microbial safety of fresh ready-to-eat vegetables produced by different technologies in Italy. *Journal of Applied Microbiology* **109**, 996-1006.

De la Noue, A.C., Estienney, M., Aho, S., Perrier-Cornet, J. M., de Rougemont, A., Pothier, P., Gervais, P. and Belliot, G. (2014) Absolute humidity influences the seasonal persistence and infectivity of human norovirus. *Applied and Environmental Microbiology* **80**, 7196-7205.

de Man, H., van den Berg, H.H.J.L., Leenen, E.J.T.M., Schijven, J.F., Schets, F.M., van der Vliet, J.C., van Knapen, F. and de Roda Husman, A.M. (2014) Quantitative assessment of infection in risk from exposure to waterborne pathogens in urban floodwater. *Water Research* **48**, 90-99.

De Roda Husman, A.M., Lodder, W.J., Rutjes, S.A., Schijven, J.F. and Teunis, P.F.M. (2009) Long-term inactivation study of three enteroviruses in artificial surface and groundwaters, using PCR and cell culture. *Applied and Environmental Microbiology* **75**, 1050-1057.

Deboosere, N., Legeay, O., Caudrelier, Y. and Lange, M. (2004) Modelling effect of physical and chemical parameters on heat inactivation kinetics of hepatitis A virus in a fruit model system. *International Journal of Food Microbiology* **93**, 73-85.

Deboosere, N., Pinon, A., Caudrelier, Y., Delobel, A., Merle, G., Perelle, S., Temmam, S., Loutreul, J., Morin, T., Estienney, M., Belliot, G., Pothier, P., Gantzer, C. and Vialette, M. (2012) Adhesion of human pathogenic enteric viruses and surrogate viruses to inert and vegetal food surfaces. *Food Microbiology* **32**, 48-56.

Deboosere, N., Pinon, A., Delobel, A., Temmam, S., Morin, T., Merle, G., Blaise-Boisseau, S., Perelle, S. and Vialette, M. (2010) A predictive microbiology approach for thermal inactivation of Hepatitis A virus in acidified berries. *Food Microbiology* **27**, 962-967.

Delaquis, P.J., Fukumoto, L.R., Toivonen, P.M.A. and Cliff, M.A. (2004) Implications of wash water chlorination and temperature for the microbiological and sensory properties of fresh-cut iceberg lettuce. *Postharvest Biology and Technology* **31**, 81-91.

Delbeke, S. (2015) Prevalence, behaviour and exposure assesment of Salmonella and Shiga toxin-producing *Escherichia coli* on basil and strawberries. PhD thesis, Ghent University, Belgium.

Dentinger, C.M., Bower, W.A., Nainan, O.V., Cotter, S.M., Myers, G., Dubusky, L.M., Fowler, S., Salehi, E.D.P. and Bell, B.P. (2001) An outbreak of hepatitis A associated with green onions. *Journal of Infectious Diseases* **183**, 1273-1276.

Devlieghere, F., debevere, J., Uyttendaele, M. and Vermeulen, A. (2011) Levensmiddelenmicrobiologie en -conservering. Brugge, Belgium: die Keure, 260 pp.

Dewaal, C.S. and Bhuiya, F. (2009) Outbreaks by the numbers: fruits and vegetables 1990-2005. *Center for Science in the Public Interest*, Available online: http://www.cspinet.org/foodsafety/IAFPPoster.pdf.

Dhanashekar, R., Akkinepalli, S. and Nellutla, A. (2012) Milk-borne infections. An analysis of their potential effect on the milk industry. *Germs* **2**, 101-109.

Di Martino, B., Ceci, C., Di Profio, F. and Marsilio, F. (2010) In vitro inactivation of feline calicivirus (FCV) by chemical disinfectants: resistance variation among field strains. *Archives of Virology* **155**, 2047-2051.

Diallo,M.B.C., Anceno,A.J., Tawatsupa,B., Houpt,E.R., Wangsuphachart,V., and Shipin,O.V. (2008) Infection risk assessment of diarrhea-related pathogens in a tropical canal network. *Science of the Total Environment* **407**, 223-232.

Ding,T., Iwahori,J., Kasuga,F., Wang,J., Forghani,F., Park,M.S., and Oh,D.H. (2013) Risk assessment for *Listeria monocytogenes* on lettuce from farm to table in Korea. *Food Control* **30**, 190-199.

Djurkovic, M. (2012) SWOT analysis of Serbia's rasberry sector in the competitive marketplace. Master Thesis. Department of economics and resource management, Norwegian University of lif sciences, 130 p. *Available online at: http://bragebibsysno/xmlui/bitstream/handle/11250/187406/Djurkovic%20Marina%202012pdf?sequence=1*.

Dolin, R., Blacklow, N.R., Dupont, H., Buscho, R.F., Wyatt, R.G., Kasel, J.A., Hornick, R. and Chanock, R.M. (1972) Biological properties of Norwalk agent of acute infectious nonbacterial gastroenteritis. *Proceedings of the Society for Experimental Biology and Medicine* **140**, 578-583.

Domenech,E., Botella,S., Ferrus,M.A., and Escriche,I. (2013) The role of the consumer in the reduction of *Listeria monocytogenes* in lettuces by washing at home. *Food Control* **29**, 98-102.

Donaldson, E.F., Lindesmith, L.C., Lobue, A.D. and Baric, R.S. (2008) Norovirus pathogenesis: mechanisms of persistence and immune evasion in human populations. *Immunological Reviews* **225**, 190-211.

Donaldson, E.F., Lindesmith, L.C., Lobue, A.D. and Baric, R.S. (2010) Viral shape-shifting: norovirus evasion of the human immune system. *Nature Reviews Microbiology* **8**, 231-241.

Drechsel,P. and Seidu,R. (2011) Cost-effectiveness of options for reducing health risks in areas where food crops are irrigated with treated or untreated wastewater. *Water International* **36**, 535-548.

Dubois, E., Agier, C., Traore, O., Hennechart, C., Merle, G., Cruciere, C. and Laveran, H. (2002) Modified concentration method for the detection of enteric viruses on fruits and vegetables by reverse transcriptase-polymerase chain reaction or cell culture. *Journal of Food Protection* **65**, 1962-1969.

Duizer, E., Bijkerk, P., Rockx, B., De Groot, A., Twisk, F. and Koopmans, M. (2004) Inactivation of caliciviruses. *Applied and Environmental Microbiology* **70**, 4538-4543.

Duizer, E., Pielaat, A., Vennema, H., Kroneman, A. and Koopmans, M. (2007) Probabilities in norovirus outbreak diagnosis. *Journal of Clinical Virology* **40**, 38-42.

Duizer, E., Schwab, K.J., Neill, F.H., Atmar, R.L., Koopmans, M.P.G. and Estes, M.K. (2004) Laboratory efforts to cultivate noroviruses. *Journal of General Virology* **85**, 79-87.

Easton,J.H., Gauthier,J.J., Lalor,M.M., and Pitt,R.E. (2005) Die-off of pathogenic *E. coli* O157 : H7 in sewage contaminated waters. *Journal of the American Water Resources Association* **41**, 1187-1193.

ECDC (2014) Outbreak of hepatitis A in EU/EEA countries - Second update, 11 April 2014. *European Centre for Disease Prevention and Control, Stockholm*.

Eden, J.S., Lim, K.L. and White, P.A. (2012) Complete genome of the human norovirus GIV.1 strain lake macquarie virus. *Journal of Virology* **86**, 10251-10252.

Edmonds, S.L., McCormack, R.R., Zhou, S.S., Macinga, D.R. and Fricker, C.M. (2012) Hand hygiene regimens for the reduction of risk in food service environments. *Journal of Food Protection* **75**, 1303-1309.

EFSA (2009) General principles for the collection of national food consumption data in view of a pan-European dietery survey. *EFSA Journal* **7**. Available online: http://www.efsa.europa.eu/en/scdocs/scdoc/1435.htm

EFSA (2010) The community summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in the European Union in 2008. *The EFSA Journal* **1496**.

EFSA (2011) EFSA Panel on Biological Hazards (BIOHAZ). Scientific opinion on irradiation of food (efficacy and microbiological safety). *EFSA Journal* **9**, 88 pp.

EFSA (2013) Scientific opinion on the risk posed by pathogens in food of non-animal origin. Part 1 (outbreak data analysis and risk ranking of food/pathogen combinations). *EFSA Journal* **11**, 138p. Available online: www.efsa.europa.eu/efsajournal.

EFSA (2014) Tracing of food items in connection to the multinational hepatitis A virus outbreak in Europe. *EFSA Journal* **12**, 186 pp.

EFSA and ECDC (2014) The European union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2012. *EFSA Journal* **12**, 312 pp.

EFSA BIOHAZ Panel, E.B. (2011) Scientific opinion on an update on the present knowledge on the occurrence and control of foodborne viruses. *EFSA Journal* **9,** doi: 10.2903/j.efsa.2011.2190. Available online: www.efsa.europa.eu/efsajournal, 96 pp.

EFSA BIOHAZ Panel, E.B. (2012a) Norovirus (NoV) in oysters: methods, limits and control options. *EFSA Journal* **10**, 10.2903/j.efsa.2012.2500. Available online: www.efsa.europa.eu/efsajournal, 39 pp.

EFSA BIOHAZ Panel, E.B. (2012b) Scientific opinion on the development of a risk ranking framework on biological hazards. *EFSA Journal* **10**, 88 pp.

EFSA BIOHAZ Panel, E.B. (2014a) Scientific opinion on the risk posed by pathogens in food of non-animal origin. Part 2 (*Salmonella* and Norovirus in berries). *EFSA Journal* **12**, 95 pp.

EFSA BIOHAZ Panel, E.B. (2014b) Scientific opinion on the risk posed by pathogens in food of non-animal origin. Part 2 (*Salmonella* and Norovirus in leafy greens eaten raw as salads). *EFSA Journal* **12**, 118 pp.

Eischeid, A.C., Thurston, J.A. and Linden, K.G. (2011) UV disinfection of adenovirus: present state of the research and future directions. *Critical Reviews in Environmental Science and Technology* **41**, 1375-1396.

El-Senousy, W.M., Costafreda, M.I., Pinto, R.M. and Bosch, A. (2013) Method validation for norovirus detectin in naturally contaminated irrigation water and fresh produce. *International Journal of Food Microbiology* **167**, 74-79.

Ensink, J. H. J., Mahgmood, T., Dalsgaard, A. (2007) Wastewater-irrigated vegetables: market handling versus irrigation water quality. *Tropical Medicine & International Health* **12**, 2-7.

Erickson, M.C. (2010) Microbial risks associated with cabbage, carrots, celery, onions, and deli salads made with these produce items. *Comprehensive Reviews in Food Science and Food Safety* **9**, 602-619.

Escudero-Abarca, B.I., Rawsthorne, H., Goulter, R.M., Suh, S.H. and Jaykus, L.A. (2014) Molecular methods used to estimate thermal inactivation of a prototype human norovirus: More heat resistant than previously believed? *Food Microbiology* **41**, 91-95.

Escudero, B.I., Rawsthorne, H., Gensel, C. and Jaykus, L.A. (2012) Persistence and transferability of noroviruses on and between common surfaces and foods. *Journal of Food Protection* **75**, 927-935.

Espinosa, A.C., Jesudhasan, P., Arredondo, R., Cepeda, M., Mazari-Hiriart, M., Mena, K.D. and Pillai, S.D. (2012) Quantifying the reduction in potential health risks by determining the sensitivity of poliovirus type 1 chat strain and rotavirus SA-11 to electron beam irradiation of iceberg lettuce and spinach. *Applied and Environmental Microbiology* **78**, 988-993.

Esseili, M.A., Wang, Q.H. and Saif, L.J. (2012a) Binding of human GII.4 norovirus virus-like particles to carbohydrates of romaine lettuce leaf cell wall materials. *Applied and Environmental Microbiology* **78**, 786-794.

Esseili, M.A., Wang, Q.H., Zhang, Z.W. and Saif, L.J. (2012b) Internalization of sapovirus, a surrogate for norovirus, in romaine lettuce and the effect of lettuce latex on virus infectivity. *Applied and Environmental Microbiology* **78**, 6271-6279.

Ethelberg, S., Lisby, M., Bottiger, B., Schultz, A.C., Villif, A., Jensen, T., Olsen, K.E., Scheutz, F., Kjelso, C. and Muller, L. (2010) Outbreaks of gastroenteritis linked to lettuce, Denmark, January 2010. *Eurosurveillance* **15**, 2-4.

Evans, M.R., Meldrum, R., Lane, W., Gardner, D., Ribeiro, C.D., Gallimore, C.I. and Westmoreland, D. (2002) An outbreak of viral gastroenteritis following environmental contamination at a concert hall. *Epidemiology and Infection* **129**, 355-360.

Falkenhorst, G., Krusell, L., Lisby, M., Madsen, S.B., Böttiger, B.E. and Molbak, K. (2005) Imported frozen raspberries cause a series of norovirus outbreaks in Denmark, 2005. *Euro Surveillance* **10**.

Fan, X., Niemira, B.A. and Prakash, A. (2008) Irradiation of fresh fruits and vegetables. *Food Technology* **62**, 36 pp.

Fao/Who (2008a) Microbiological hazards in fresh leafy vegetables and herbs: Meeting Report. *Microbiological Risk Assessment Series* **14**, 151.

Fao/Who (2008b) Viruses in food: scientific advice to support risk management activities, meeting report. *Microbiological Risk Assessment series no13 Rome*.

FAO/WHO (2008c) Microbiological hazards in fresh fruits and vegetables. *Microbiological risk assessment series, Meeting report* 1-28.

FAO/WHO (2012) Guidelines on the application of general principles of food hygiene to the control of viruses in food, CAC/GL 79-2012. *Available from wwwcodexalimentariusorg/standards/list-of-standards/ (last accessed on 2014-03-31)*.

Farkas, T., Sestak, K., Wei, C. and Jiang, X. (2008) Characterization of a rhesus monkey calicivirus representing a new genus of Caliciviridae. *Journal of Virology* **82**, 5408-5416.

FAVV (2014) EFSA Belgian reports on zoonoses and zoonotic agents 2004-2012. *available online: http://wwwfavv-afscafgovbe/thematischepublicaties/efsa-bel-report-zoonoses-zoonotic-agentsasp*

FDA (2001) Food and drug administration, center for food safety and applied nutrition. In Evaluation and definition of potentially hazardous foods. Chapter 7. Comparison of NSF and ABA protocols to determine whether a food requires time/temperature control for safety. Available http://www.fda.gov/Food/FoodScienceResearch/SafePracticesforFoodProcesses/ucm094156.htm.

FDA (2008) Irradiation in the production, processing and handling of food. . *Federal Register* **73**, 11.

Feng, K., Divers, E., Ma, Y.M. and Li, J.R. (2011) Inactivation of a human norovirus surrogate, human norovirus virus-like particles, and vesicular stomatitis virus by gamma irradiation. *Applied and Environmental Microbiology* **77**, 3507-3517.

Ferrer,A., Nguyen-Viet,H., and Zinsstag,J. (2012) Quantification of diarrhea risk related to wastewater contact in Thailand. *Ecohealth* **9**, 49-59.

Ferrier, P. and Buzby, J.C. (2014) The limits of food safety testing: A case study of Escherichia coli testing of beef trim. *Food Control* **38**, 227-234.

Fewtrell, L., Bartram, J. (2001) Water quality: Guidelines, standards and health - Assessment of risk and risk management for water-related infectious disease. World Health Organization, IWA Publishing, London, UK.

Finley,S., Barrington,S., and Lyew,D. (2009) Reuse of domestic greywater for the irrigation of food crops. *Water Air and Soil Pollution* **199**, 235-245.

Fino, V.R. and Kniel, K.E. (2008) Comparative recovery of foodborne viruses from fresh produce. *Foodborne Pathogens and Disease* **5**, 819-825.

Fisher, I.S. and O'Brien, S. (2001) *Salmonella* newport infection in England associated with the consumption of ready to eat salad. *Eurosurveillance* **5**.

Fogarty, L.R., Haack, S.K., Wolcott, M.J. and Whitman, R.L. (2003) Abundance and characteristics of the recreational water quality indicator bacteria *Escherichia coli* and *enterococci* in gull faeces. *Journal of Applied Microbiology* **94**, 865-878.

Fong, T.T. and Lipp, E.K. (2005) Enteric viruses of humans and animals in aquatic environments: Health risks, detection, and potential water quality assessment tools. *Microbiology and Molecular Biology Reviews* **69**, 357-371.

Forslund,A., Ensink,J.H.J., Battilani,A., Kljujev,I., Gola,S., Raicevic,V., Jovanovic,Z., Stikic,R., Sandei,L., Fletcher,T., and Dalsgaard,A. (2010) Faecal contamination and hygiene aspect associated with the use of treated wastewater and canal water for irrigation of potatoes (*Solanum tuberosum*). *Agricultural Water Management* **98**, 440-450.

Forslund,A., Ensink,J.H.J., Markussen,B., Battilani,A., Psarras,G., Gola,S., Sandei,L., Fletcher,T., and Dalsgaard,A. (2012) Escherichia coli contamination and health aspects of soil and tomatoes (*Solanum lycopersicum L.*) subsurface drip irrigated with on-site treated domestic wastewater. *Water Research* **46**, 5917-5934.

Fout, G.S., Brinkman, N.E., Cashdollar, J.L., Griffin, S.M., McMinn, B.R., Rhodes, E.R., Varughese, E.A., Karim, M.R., Grimm, A.C., Spencer, S.K. and Borchardt, M.A. (2010) Method 1615:

measurement of enterovirus and norovirus occurrence in water by culture and Rt-qPCR, publicaton no EPA/600/R-10/181. *US Environmental Protection Agency, Cincinnati, OH*.

Fout, G.S., Martinson, B.C., Moyer, M.W.N. and Dahling, D.R. (2003) A multiplex reverse transcription-PCR method for detection of human enteric viruses in groundwater. *Applied and Environmental Microbiology* **69**, 3158-3164.

Fraisse, A., Temmam, S., Deboosere, N., Guillier, L., Delobel, A., Maris, P., Vialette, M., Morin, T. and Perelle, S. (2011) Comparison of chlorine and peroxyacetic-based disinfectant to inactivate Feline calicivirus, Murine norovirus and Hepatitis A virus on lettuce. *International Journal of Food Microbiology* **151**, 98-104.

Franck, K.T., Lisby, M., Fonager, J., Schultz, A.C., Böttiger, B., Villif, A., Absalonsen, H. and Ethelberg, S. (2015) Sources of calicivirus contamination in foodborne outbreaks in Denmark, 2005-2011 – The role of the asymptomatic food handler. *The Journal of Infectious Diseases* **211**, 563-570.

Franz,E., Tromp,S.O., Rijgersberg,H., and Fels-Klerx,H.J. (2010) Quantitative microbial risk assessment for *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in leafy green vegetables consumed at salad bars. *Journal of Food Protection* **73**, 274-285.

Friesema, I., Schimmer, B., Stenvers, O., Heuvelink, A., de Boer, E., van der Zwaluw, K., de Jager, C., Notermans, D., van Ouwerkerk, I., de Jonge, R. and van Pelt, W. (2007) STEC O157 outbreak in the Netherlands, September-October 2007. *Eurosurveillance* **12**.

Friesema, I., Sigmundsdottir, G., Van der Zwaluw, K., Heuvelink, A., Schimmer, B., de Jager, C., Rump, B., Briem, H., Hardardottir, H., Atladottir, A., Gudmundsdottir, E. and Van Pelt, W. (2008) An international outbreak of shiga toxin-producing *Escherichia coli* O157 infection due to lettuce, september - october 2007. *Eurosurveillance* **13**.

FSAI (2001) Code of practice for food safety in the fresh produce supply chain in Ireland. Dublin: Food Safety Authorities of Ireland.

Fumian, T.M., Leite, J.P.G., Castello, A.A., Gaggero, A., de Caillou, M.S.L. and Miagostovich, M.P. (2010) Detection of rotavirus A in sewage samples using multiplex qPCR and an evaluation of the ultracentrifugation and adsorption-elution methods for virus concentration. *Journal of Virological Methods* **170**, 42-46.

Gabrieli, R., Maccari, F., Ruta, A., Pana, A. and Divizia, M. (2009) Norovirus detection in groundwater. *Food and Environmental Virology* **1**, 92-96.

Gandhi, K.M., Mandrell, R.E. and Tian, P. (2010) Binding of virus-like particles of Norwalk virus to romaine lettuce veins. *Applied and Environmental Microbiology* **76**, 7997-8003.

Gassilloud, B., Duval, M., Schwartzbrod, L. and Gantzer, C. (2003) Recovery of feline calicivirus infectious particles and genome from water: comparison of two concentration techniques. *Water Science and Technology* **47**, 97-101.

Gaulin, C., Frigon, M., Poirier, D. and Fournier, C. (1999a) Transmission of calicivirus by a foodhandler in the pre-symptomatic phase of illness. *Epidemiology and Infection* **123**, 475-478.

Gaulin, C.D., Ramsay, D., Cardinal, P. and D'Halevyn, M.A. (1999b) Viral gastroenteritis epidemic associated with the ingestion of imported raspberries. *Canadian Journal of Public Health-Revue Canadienne de Sante Publique* **90**, 37-40.

Gensberger, E.T. and Kostic, T. (2013) Novel tools for environmental virology. *Current Opinion in Virology* **3**, 61-68.

Gerba, C.P. (2007) Virus occurrence and survival in the environmental waters. In *Human viruses in water* ed. Bosch, A. p.299. Amsterdam, The Netherlands: Elsevier.

Gerba, C.P. (2009) The role of water and water testing in produce safety. In *Microbial safety of fresh produce* eds. Fan, X., Niemira, B.A., Doona, C.J., Feeherry, F.E. and Gravani, R.B.: Wiley-Blackwell.

Gibbons, C.D., Rodriguez, R.A., Tallon, L. and Sobsey, M.D. (2010) Evaluation of positively charged alumina nanofibre cartridge filters for the primary concentration of noroviruses, adenoviruses and male-specific coliphages from seawater. *Journal of Applied Microbiology* **109**, 635-641.

Gibney,K., Sinclair,M., O'Toole,J., and Leder,K. (2013) Using disability-adjusted life years to set health-based targets: A novel use of an established burden of disease metric. *Journal of Public Health Policy* **34**, 439-446.

Gibson, K.E., Crandall, P.G. and Ricke, S.C. (2012) Removal and transfer of viruses on food contact surfaces by cleaning cloths. *Applied and Environmental Microbiology* **78**, 3037-3044.

Gibson, K.E. and Schwab, K.J. (2011) Tangential-flow ultrafiltration with integrated inhibition detection for recovery of surrogates and human pathogens from large-volume source water and finished drinking water. *Applied and Environmental Microbiology* **77**, 385-391.

Gil, M.I., Selma, M.V., Lopez-Galvez, F. and Allende, A. (2009) Fresh-cut product sanitation and wash water disinfection: Problems and solutions. *International Journal of Food Microbiology* **134**, 37-45.

Gillesber Lassen, S., Soborg, B., Midgley, S.E., Steens, A., Vold, L., Stene-Johansen, K., Rimhanen-Finne, R., Kontio, M., L"fdahl, M., Sundqvist, L., Edelstein, M., Jensen, T., Vestergaard, H.T., Fischer, T.K., Molbak, K. and Ethelberg, S. (2013) Ongoing multi-strain food-borne hepatitis A outbreak with frozen berries as suspected vehicle: four Nordic countries affected, October 2012 to April 2013. *Euro Surveillance* **18**.

Girard, M., Morales-Rayas, R. and Jean, J. (2013) Comparison of RNA Extraction Methods for the Detection of a Norovirus Surrogate in Ready-to-Eat Foods. *Foodborne Pathogens and Disease* **10**, 47-54.

Girard, M., Ngazoa, S., Mattison, K. and Jean, J. (2010) Attachment of noroviruses to stainless steel and their inactivation, using household disinfectants. *Journal of Food Protection* **73**, 400-404.

Glass, R.I., Parashar, U.D. and Estes, M.K. (2009) CURRENT CONCEPTS Norovirus gastroenteritis. *New England Journal of Medicine* **361**, 1776-1785.

Gomes, K.A., Stupka, J.A., Gomez, J. and Parra, G.I. (2007) Molecular characterization of calicivirus strains detected in outbreaks of gastroenteritis in Argentina. *Journal of Medical Virology* **79**, 1703-1709.

Gomez-Aldapa, C.A., Rangel-Vargas, E., Bautista-De Leon, H., Vazquez-Barrios, M.E., Gordillo-Martinez, A.J. and Castro-Rosas, J. (2013) Behavior of enteroaggregative *Escherichia coli*, non-O157-shiga toxin-producing *E-coli*, enteroinvasive *E-coli*, enteropathogenic *E-coli* and enterotoxigenic *E-coli* strains on mung bean seeds and sprout. *International Journal of Food Microbiology* **166**, 364-368.

Gomez-Lopez, V.M., Marin, A., Medina-Martinez, M.S., Gil, M.I. and Allende, A. (2013) Generation of trihalomethanes with chlorine-based sanitizers and impact on microbial, nutritional and sensory quality of baby spinach. *Postharvest Biology and Technology* **85**, 210-217.

Goodburn, C. and Wallace, C.A. (2013) The microbiological efficacy of decontamination methodologies for fresh produce: A review. *Food Control* **32**, 418-427.

Gotz, H., De Jong, B., Lindback, J., Parment, P.A., Hedlund, K.O., Torven, M. and Ekdahl, K. (2002) Epidemiological investigation of a food-borne gastroenteritis outbreak caused by Norwalk-like virus in 30 day-care centres. *Scandinavian Journal of Infectious Diseases* **34**, 115-121.

Goyal, S.M. (2006) Viruses in food. New York: Springer Science.

Goyal, S.M. and Gerba, C.P. (1979) Comparative adsorption of human enteroviruses, simian rotavirus, and selected bacteriphages to soils. *Applied and Environmental Microbiology* **38**, 241-247.

Graham, D.Y., Jiang, X., Tanaka, T., Opekun, A.R., Madore, H.P. and Estes, M.K. (1994) Norwalk virus-infection of volunteers – new insights based on improved assays. *Journal of Infectious Diseases* **170**, 34-43.

Gray, J.J., Kohli, E., Ruggeri, F.M., Vennema, H., Sanchez-Fauquier, A., Schreier, E., Gallimore, C.I., Iturriza-Gomara, M., Giraudon, H., Pothier, P., Di Bartolo, I., Inglese, N., de Bruin, E., van der Veer, B., Moreno, S., Montero, V., de Llano, M.C., Hoehne, M. and Diedrich, S.M. (2007) European multicenter evaluation of commercial enzyme immunoassays for detecting norovirus antigen in fecal samples. *Clinical and Vaccine Immunology* **14**, 1349-1355.

Green, J., Wright, P.A., Gallimore, C.I., Mitchell, O., Morgan-Capner, P. and Brown, D.W.G. (1998) The role of environmental contamination with small round structured viruses in a hospital outbreak investigated by reverse-transcriptase polymerase chain reaction assay. *Journal of Hospital Infection* **39**, 39-45.

Green, K.Y., Ando, T., Balayan, M.S., Berke, T., Clarke, I.N., Estes, M.K., Matson, D.O., Nakata, S., Neill, J.D., Studdert, M.J. and Thiel, H.J. (2000) Taxonomy of the caliciviruses. *Journal of Infectious Diseases* **181**, S322-S330.

Griffin, M.R., Surowiec, J.J., McCloskey, D.I., Capuano, B., Pierzynski, B., Quinn, M., Wojnarski, R., Parkin, W.E., Greenberg, H. and Gary, G.W. (1982) Foodborne Norwalk virus. *American Journal of Epidemiology* **115**, 178-184.

Grimason,A.M., Smith,H.V., Thitai,W.N., Smith,P.G., Jackson,M.H., and Girdwood,R.W.A. (1993) Occurrence and removal of *Cryptosporidium* spp Oocysts and *Giardia* spp cysts in Kenyan waste stabilization ponds. *Water Science and Technology* **27**, 97-104.

Grotto, I., Huerta, M., Balicer, R.D., Halperin, T., Cohen, D., Orr, N. and Gdalevich, M. (2004) An outbreak of norovirus gastroenteritis on an Israeli military base. *Infection* **32**, 339-343.

Guzman-Herrador, B., Jensvoll, L., Einöder-Moreno, M., Lange, M., Myking, S., Nygard, K., Stene-Johansen, K. and Vold, L. (2014) Ongoing hepatitis A outbreak in Europe 2013 to 2014: imported berry mix cake suspected to be the source of infection in Norway. *Euro Surveillance* **19**, Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20775.

Haack, S.K., Fogarty, L.R. and Wright, C. (2003) *Escherichia coli* and *enterococci* at beaches in the Grand Traverse Bay, Lake Michigan: Sources, characteristics, and environmental pathways. *Environmental Science & Technology* **37**, 3275-3282.

Haas, C. N. (1983) Effect of effluent disinfection on risks of viral disease transmission via recreational water exposure. *Journal Water Pollution Control Federation* **55**, 1111-1116.

Haas,C.N. (1996) Acceptable microbial risk. *Journal American Water Works Association* **88**, 8.

Haas,C.N., Rose,J.B., Gerba,C., and Regli,S. (1993) Risk assessment of virus in drinking-water. *Risk Analysis* **13**, 545-552.

Haas,C.N., Rose,J.B. and Gerba,C.P. (1999) *Quantitative Microbial Risk Assessment*, John Wiley & Sons, West Sussex, England, 464 pp.

Hall, A.J., Lopman, B., Park, G.W., Yen, C., Gregoricus, N. and Parashar, U.D. (2011) Updated norovirus outbreak management and disease prevention guidelines. *Morbidity and Mortality Weekly Report Recommendations and Reports* **60**, 18 pp.

Hall, A.J., Wikswo, M.E., Pringle, K., Gould, L.H. and Parashar, U.D. (2014) Vital Signs: Foodborne norovirus outbreaks - United States, 2009-2012. *Mmwr-Morbidity and Mortality Weekly Report* **63**, 491-495.

Hamilton, A.J., Stagnitti, F., Premier, R., Boland, A.M. and Hale, G. (2006) Quantitative microbial risk assessment models for consumption of raw vegetables irrigated with reclaimed water. *Applied and Environmental Microbiology* **72**, 3284-3290.

Hamilton,A.J., Stagnitti,F., Xiong,X.Z., Kreidl,S.L., Benke,K.K., and Maher,P. (2007) Wastewater irrigation: The state of play. *Vadose Zone Journal* **6**, 823-840.

Hamilton,A.J., Stagnitti,F.S., Premier,R., and Boland,A.M. (2006b) Is the risk of illness through consuming vegetables irrigated with reclaimed wastewater different for different population groups? *Water Science and Technology* **54**, 379-386.

Hamza, I.A., Jurzik, L., Stang, A., Sure, K., Uberla, K. and Wilhelm, M. (2009) Detection of human viruses in rivers of a densly-populated area in Germany using a virus adsorption elution method optimized for PCR analyses. *Water Research* **43**, 2657-2668.

Han, T.H., Kim, S.C., Kim, S.T., Chung, C.H. and Chung, J.Y. (2014) Detection of norovirus genogroup IV, klassevirus, and pepper mild mottle virus in sewage samples in South Korea. *Archives of Virology* **159**, 457-463.

Han, Y., Linton, R.H., Nielsen, S.S. and Nelson, P.E. (2000a) Inactivation of *Escherichia coli* O157 : H7 on surface-uninjured and -injured green pepper (*Capsicum annuum L*.) by chlorine dioxide gases demonstrated by confocal laser scanning microscopy. *Food Microbiology* **17**, 643-655.

Han, Y., Sherman, D.M., Linton, R.H., Nielsen, S.S. and Nelson, P.E. (2000b) The effects of washing and chlorine dioxide gas on survival and attachment of *Escherichia coli* O157 : H7 to green pepper surfaces. *Food Microbiology* **17**, 521-533.

Haramoto, E., Katayama, H., Oguma, K. and Ohgaki, S. (2005) Application of cation-coated filter method to detection of noroviruses, enteroviruses, adenoviruses, and torque teno viruses in the Tamagawa River in Japan. *Applied and Environmental Microbiology* **71**, 2403-2411.

Haramoto, E., Katayama, H., Oguma, K. and Ohgaki, S. (2007) Recovery of naked viral genomes in water by virus concentration methods. *Journal of Virological Methods* **142**, 169-173.

Haramoto, E., Katayama, H., Utagawa, E. and Ohgaki, S. (2009) Recovery of human norovirus from water by virus concentration methods. *Journal of Virological Methods* **160**, 206-209.

Harris, L.J., Farber, J.N., Beuchat, L.R., Parish, M.E., Suslow, T.V., Garrett, E.H. and Busta, F.F. (2003) Outbreaks associated with fresh produce: incidence, growth, and survival of pathogens in fresh and fresh-cut produce. *Comprehensive Reviews in Food Science and Food Safety* **2**, 78-131.

Hartmann, E.M. and Halden, R.U. (2012) Analytical methods for the detection of viruses in food by example of CCL-3 bioagents. *Analytical and Bioanalytical Chemistry* **404**, 2527-2537.

Hata, A., Katayama, H., Kitajima, M., Visvanathan, C., Nol, C. and Furumai, H. (2011) Validation of internal controls for extraction and amplification of nucleic acids from enteric viruses in water samples. *Applied and Environmental Microbiology* **77**, 4336-4343.

Havelaar, A.H., Haagsma, J.A., Mangen, M.J.J., Kemmeren, J.M., Verhoef, L.P.B., Vijgen, S.M.C., Wilson, M., Friesema, I.H.M., Kortbeek, L.M., van Duynhoven, Y.T.H.P. and van Pelt, W. (2012) Disease burden of foodborne pathogens in the Netherlands, 2009. *International Journal of Food Microbiology* **156**, 231-238.

Havelaar, A. H. and Melse, J. M. (2003) Quantifying public health risk in the WHO guidelines for drinking-water quality: a burden of disease approach, RIVM report 734301022. Rijksinstituut voor Volksgezondheid en Milieu, The Netherlands

He,X.Q., Wei,Y.M., Cheng,L., Zhang,D.Y., and Wang,Z.J. (2012) Molecular detection of three gastroenteritis viruses in urban surface waters in Beijing and correlation with levels of fecal indicator bacteria. *Environmental Monitoring and Assessment* **184**, 5563-5570.

Hedberg, C.W. (2006) Epidemiology of viral food-borne outbreaks. In *Viruses in foods* ed. Goyal, S.M. p.345. United States of America: Springer.

Hedin, G., Rynback, J. and Lore, B. (2010) New technique to take samples from environmental surfaces using flocked nylon swabs. *Journal of Hospital Infection* **75**, 314-317.

Hernandez, F., Monge, R., Jimenez, C. and Taylor, L. (1997) Rotavirus and Hepatitis A virus in market lettuce (*Latuca sativa*) in Costa Rica. *International Journal of Food Microbiology* **37**, 221-223.

Herwaldt, B.L., Ackers, M.L., Farrar, J., Richardson, S., Nelson, R., Fletcher, M., Levy, M., Katz, D., Kumar, S., Malecki, J., Lowdermilk, M., Mackey, L., Bell, J., Portesi, D., Lacey, C., Letendre, L., Hamlin, D., Knowlton, R., Barry, A., Chew, D., Finelli, L., Genese, C., Miller, J., Layton, M., Guzewich, J., Salehi, E., Weltman, A., Caceres, V., Ball, R., Barnett, B., Hendricks, K., Taylor, J., Bell, R., Schoenfeld, S., Bryan, F., Neamatullah, S., Werker, D., Manuel, D., Leber, C., Arrieta, M., Morrison, D., Klein, R., Wahlquist, S., Alfano, E., Eberhard, M., Arrowood, M., HannakDonaldson, K., Beach, M., Kramer, M., Hightower, A., Swerdlow, D., Winickoff, J., Shapiro, R. and Messonnier, M. (1997) An outbreak in 1996 of cyclosporiasis associated with imported raspberries. *The New England Journal of Medicine* **336**, 1548-1556.

Herzog, A.B., Pandey, A.K., Reyes-Gastelum, D., Gerba, C.P., Rose, J.B. and Hashsham, S.A. (2012) Evaluation of sample recovery efficiency for Bacteriophage P22 on vomites. *Applied and Environmental Microbiology* **78**, 7915-7922.

Hewitt, J. and Greening, G.E. (2006) Effect of heat treatment on hepatitis A virus and norovirus in New Zealand greenshell mussels (*Perna canaliculus*) by quantitative real-time reverse transcription PCR and cell culture. *Journal of Food Protection* **69**, 2217-2223.

Hewitt, J., Leonard, M., Greening, G.E. and Lewis, G.D. (2011) Influence of wastewater treatment process and the population size on human virus profiles in wastewater. *Water Research* **45**, 6267-6276.

Hida, K., Kulka, M. and Papafragkou, E. (2013) Development of a rapid total nucleic acid extraction method for the isolation of hepatitis A virus from fresh produce. *International Journal of Food Microbiology* **161**, 143-150.

Hijnen, W.A.M., Beerendonk, E.F. and Medema, G.J. (2006) Inactivation credit of UV radiation for viruses, bacteria and protozoan (oo)cysts in water: A review. *Water Research* **40**, 3-22.

Hijnen, W.A.M., Brouwer-Hanzens, A.J., Charles, K.J. and Medema, G.J. (2005) Transport of MS2 phage, *Escherichia coli*, *Clostridium perfringens*, *Cryptosporidium parvum* and *Giardia intestinalis* in a gravel and a sandy soil. *Environmental Science & Technology* **39**, 7860-7868.

Hirneisen, K.A., Black, E.P., Cascarino, J.L., Fino, V.R., Hoover, D.G. and Kniel, K.E. (2010) Viral inactivation in foods: A review of traditional and novel food-processing technologies. *Comprehensive Reviews in Food Science and Food Safety* **9**, 3-20.

Hirneisen, K.A. and Kniel, K.E. (2013a) Comparative uptake of enteric viruses into spinach and green onions. *Food and Environmental Virology* **5**, 24-34.

Hirneisen, K.A. and Kniel, K.E. (2013b) Inactivation of internalized and surface contaminated enteric viruses in green onions. *International Journal of Food Microbiology* **166**, 201-206.

Hirneisen, K.A. and Kniel, K.E. (2013c) Norovirus surrogate survival on spinach during preharvest growth. *Phytopathology* **103**, 389-394.

Hirneisen, K.A., Markland, S.M. and Kniel, K.E. (2011) Ozone inactivation of norovirus surrogates on fresh produce. *Journal of Food Protection* **74**, 836-839.

Hirneisen, K.A., Sharma, M. and Kniel, K.E. (2012) Human enteric pathogen internalization by root uptake into food crops. *Foodborne Pathogens and Disease* **9**, 396-405.

Hjertqvist, M., Johansson, A., Svensson, N., Abom, P.E., Magnusson, C., Olsson, M., Hedlund, K.O. and Andersson, Y. (2006) Four outbreaks of norovirus gastroenteritis after consuming raspberries, Sweden, June-August 2006. *Euro Surveillance* **11**.

Hoelzer, K., Fanaselle, W., Pouillot, R., Van Doren, J.M. and Dennis, S. (2013) Virus inactivation on hard surfaces or in suspension by chemical disinfectants: systematic review and meta-analysis of norovirus surrogates. *Journal of Food Protection* **76**, 1006-1016.

Hoelzer, K. Pouillot, R., Egan, K., and Dennis, S. (2012) Produce consumption in the United States: An analysis of consumption frequencies, serving sizes, processing forms, and high-consumping population subgroups for microbial risk assessment. *Journal of Food Protection* **75**, 328-340.

Hoglund,C., Stenstrom,T.A., and Ashbolt,N. (2002) Microbial risk assessment of source-separated urine used in agriculture. *Waste Management & Research* **20**, 150-161.

Holvoet, K. (2014) Bacterial safety of lettuce in primary production and fresh-cut processing industry. PhD thesis, Ghent University, Belgium.

Holvoet, K., De Keuckelaere, A., Sampers, I., Van Haute, S., Stals, A. and Uyttendaele, M. (2014) Quantitative study of cross-contamination with *Escherichia coli*, *E. coli* O157, MS2 phage and murine norovirus in a simulated fresh-cut lettuce wash process. *Food Control* **37**, 218-227.

Holvoet, K., Jacxsens, L., Sampers, I. and Uyttendaele, M. (2012) Insight into the prevalence and distribution of microbial contamination to evaluate water management in the fresh produce processing industry. *Journal of Food Protection* **75**, 671-681.

Horby, P.W., O'Brien, S.J., Adak, G.K., Graham, C., Hawker, J.I., Hunter, P., Lane, C., Lawson, A.J., Mitchell, R.T., Reacher, M.H., Threlfall, E.J. and Ward, L.R. (2003) A national outbreak of multi-resistant Salmonella enterica serovar Typhimurium definitive phage type (DT) 104 associated with consumption of lettuce. *Epidemiology and Infection* **130**, 169-178.

Horm, K.M. and D'Souza, D.H. (2011) Survival of human norovirus surrogates in milk, orange, and pomegranate juice, and juice blends at refrigeration (4 degrees C). *Food Microbiology* **28**, 1054-1061.

Horm, K.M., Davidson, P.M., Harte, F.M. and D'Souza, D.H. (2012a) Survival and inactivation of human norovirus surrogates in blueberry juice by high-pressure homogenization. *Foodborne Pathogens and Disease* **9**, 974-979.

Horm, K.M., Harte, F.M. and D'Souza, D.H. (2012b) Human norovirus surrogate reduction in milk and juice blends by high pressure homogenization. *Journal of Food Protection* **75**, 1984-1990.

Howard, G., Pedley, S., and Tibatemwa, S. (2006) Quantitative microbial risk assessment to estimate health risks attributable to water supply: can the technique be applied in developing countries with limited data? *Journal of Water and Health* **4**, 49-65.

Hsu, W.Y., Simonne, A., Jitareerat, P. and Marshall, M.R. (2010) Low-dose irradiation improves microbial quality and shelf life of fresh mint (*Mentha piperita L.*) without compromising visual quality. *Journal of Food Science* **75**, M222-M230.

Hunt, R.J., Coplen, T.B., Haas, N.L., Saad, D.A. and Borchardt, M.A. (2005) Investigating surface water-well interaction using stable isotope ratios of water. *Journal of Hydrology* **302**, 154-172.

Hurst, C.J., Gerba, C.P. and Cech, I. (1980) Effects of environmental variables and soil characteristics on virus survival in soil. *Applied and Environmental Microbiology* **40**, 1067-1079.

Hutin, Y.J.F., Pool, V., Cramer, E.H., Nainan, O.V., Weth, J., Williams, I.T., Goldstein, S.T., Gensheimer, K.F., Bell, B.P., Shapiro, C.N., Alter, M.J. and Margolis, H.S. (1999) A multistate, foodborne outbreak of hepatitis A. *New England Journal of Medicine* **340**, 595-602.

ICMSF (2011) (International Commission on Microbiological Specifications for Foods). Microorganisms in Food 8: Use of data for assessing process control and product acceptance. Springer, New York.

IFT/FDA (2001) Analysis & evaluation of preventive control measures for the control & reduciton/elimination ofmicrobial hazards on fresh & fresh-cut produce. Chapter VII. The use of indicators and surrogate microorganisms for the evaluation of pathogens in fresh and fresh-cut produce. A report of the Institute of Food Technologists for the Food and Drug Administration of the United States, Department of Health and Human Services Available online: http://wwwfdagov/Food/FoodScienceResearch/SafePracticesforFoodProcesses/ucm090977htm (last accessed on 26th October 2014).

Iglesias, R., Ortega, E., Batanero, G., Quintas, L. (2010) Water reuse in Spain: data overview and costs estimation of suitable treatment trains. *Desalination* **263**, 1-10.

Ikner, L.A., Gerba, C.P. and Bright, K.R. (2012) Concentration and recovery of viruses from water: a comprehensive review. *Food and Environmental Virology* **4**, 41-67.

Ikner, L.A., Soto-Beltran, M. and Bright, K.R. (2011) New method using a positively charged microporous filter and ultrafiltration for concentration of viruses from tap water. *Applied and Environmental Microbiology* **77**, 3500-3506.

Ilic,S., Rajic,A., Britton,C.J., Grasso,E., Wilkins,W., Totton,S., Wilhelm,B., Waddell,L., and LeJeune,J.T. (2012) A scoping study characterizing prevalence, risk factor and intervention research, published between 1990 and 2010, for microbial hazards in leafy green vegetables. *Food Control* **23**, 7-19.

Ingram, M. (1977) The significance of the index and indicator organisms in foods. Presented at the Tenth International Symposium of the IUMS Committee on Food Microbiology and Hygiene, held at Szczecin, Poland, September 5-10, 1977. Unpublished: for an account of this paper, see Mossel, D. A. A., Coliform test for cheese and other foods. *Lancet* **2**, 1425.

Iritani, N., Vennema, H., Siebenga, J.J., Siezen, R.J., Renckens, B., Seto, Y., Kaida, A. and Koopmans, M. (2008) Genetic analysis of the capsid gene of genotype GII.2 noroviruses. *Journal of Virology* **82**, 10.

Isakbaeva, E.T., Widdowson, M.A., Beard, R.S., Bulens, S.N., Mullins, J., Monroe, S.S., Bresee, J., Sassano, P., Cramer, E.H. and Glass, R.I. (2005) Norovirus transmission on cruise ship. *Emerging Infectious Diseases* **11**, 154-157.

Jacxsens, L., Castro Ibanez, I., Gomez-Lopez, V. M., Araujo Fernandes, J., Allende, A., Uyttendaele, M., and Huybrechts, I. (submitted) Belgian and Spanish consumption data and consumer handling practices of fresh fruits and vegetables useful for further microbiological and chemical exposure assessment. *Journal of Food Protection*.

Jin, M., He, Y.Q., Li, H.Y., Huang, P.W., Zhong, W.M., Yang, H., Zhang, H.L., Tan, M. and Duan, Z.J. (2013) Two gastroenteritis outbreaks caused by GII noroviruses: Host susceptibility and HBGA phenotypes. *Plos One* **8**.

Jin, Y. and Flury, M. (2002) Fate and transport of viruses in porous media. *Advances in Agronomy* **77**, 39-102.

John, D.E. and Rose, J.B. (2005) Review of factors affecting microbial survival in groundwater. *Environmental Science & Technology* **39**, 7345-7356.

Jones, T.H., Muehlhauser, V. and Croken, G. (2012) Development of an optimized method for the recovery of infectious F-RNA coliphage MS2 from meat. *Journal of Virological Methods* **185**, 69-73.

Jones, T.H., Muehlhauser, V. and Thériault, G. (2014) Comparison of ZetaPlus 60S and nitrocellulose membrane filters for the simultaneous concentation of F-RNA coliphages, porcine teschovirus and porcine adenovirus from river water. *Journal of Virological Methods* **206**, 5-11.

Jones, M. K., Watanabe, M., Zhu, S., Graves, C. L., Keyes, L. R., Grau, K. R., Gonzalez-Hernandez, M. B., Iovine, N. M., Wobus, C. E., Vinjé, J., Tibbetts, S. A., Wallet, S. M. and Karst, S. M. (2014) Enteric bacteria promote human and mouse norovirus infection of B cells. *Science* **346**, 755-759.

Jothikumar, N., Kang, G. and Hill, V.R. (2009) Broadly reactive TaqMan (R) assay for real-time RT-PCR detection of rotavirus in clinical and environmental samples. *Journal of Virological Methods* **155**, 126-131.

Jothikumar, N., Lowther, J.A., Henshilwood, K., Lees, D.N., Hill, V.R. and Vinje, J. (2005) Rapid and sensitive detection of noroviruses by using TaqMan-based one-step reverse transcription-PCR assays

and application to naturally contaminated shellfish samples. *Applied and Environmental Microbiology* **71**, 1870-1875.

Julian, T.R., Tamayo, F.J., Leckie, J.O. and Boehm, A.B. (2011) Comparison of surface sampling methods for virus recovery from fomites. *Applied and Environmental Microbiology* **77**, 6918-6925.

Jurzik, L., Hamza, I.A., Puchert, W., Uberla, K. and Wilhelm, M. (2010) Chemical and microbiological parameters as possible indicators for human enteric viruses in surface water. *International Journal of Hygiene and Environmental Health* **213**, 210-216.

Kapikian, A.Z. (2000) The discovery of the 27-nm Norwalk virus: An historic perspective. *Journal of Infectious Diseases* **181**, S295-S302.

Kapikian, A.Z., Wyatt, R.G., Dolin, R., Thornhil.Ts, Kalica, A.R. and Chanock, R.M. (1972) Visualization by immune electro-microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. *Journal of Virology* **10**, 1075-1081.

Kaplan, J.E., Feldman, R., Campbell, D.S., Lookabaugh, C. and Gary, G.W. (1982) The frequency of a Norwalk-like pattern of illness in outbreaks of acute gastroenteritis. *American Journal of Public Health* **72**, 1329-1332.

Karst, S.M., Wobus, C.E., Lay, M., Davidson, J. and Virgin, H.W. (2003) STAT1-dependent innate immunity to a Norwalk-like virus. *Science* **299**, 1575-1578.

Kartst, S.M., Zhu, S. and Goodfellow, I.G. (2015) The molecular pathology of noroviruses. *Journal of Pathology* **235**, 206-216.

Katayama, H., Shimasaki, A. and Ohgaki, S. (2002) Development of a virus concentration method and its application to detection of enterovirus and Norwalk virus from coastal seawater. *Applied and Environmental Microbiology* **68**, 1033-1039.

Kayed, D. (2004) Microbial quality of irrigation water used in the production of fresh produce in Arizona. . In *department of microbiology and immunology*. p.151. Tucson, AZ: University of Arizona.

Kelland, K. (2012) Vomiting Larry battles "Ferrari of the virus world". London, Reuters. Available online: http://www.reuters.com/article/2012/12/31/us-norovirus-idUSBRE8BU05N20121231

Keskinen, L.A. and Annous, B.A. (2011) Efficacy of adding detergents to sanitizer solutions for inactivation of *Escherichia coli* O157:H7 on romaine lettuce. *International Journal of Food Microbiology* **147**, 157-161.

Kim, A.-N., Park, S.Y., Bae, S.C., Oh, M.H. and Ha, S.D. (2014) Survival of norovirus surrogate on various food-contact surfaces. *Food and Environmental Virology* **6**, 182-188.

Kim, S.J., Si, J., Lee, J.E. and Ko, G. (2012) Temperature and humidity influences on inactivation kinetics of enteric viruses on surfaces. *Environmental Science & Technology* **46**, 13303-13310.

Kingsley, D.H. (2013) High pressure processing and its application to the challenge of virus-contaminated food. *Food and Environmental Virology* **5**, 1-12.

Kingsley, D.H. and Chen, H.Q. (2009) Influence of pH, salt, and temperature on pressure inactivation of hepatitis A virus. *International Journal of Food Microbiology* **130**, 61-64.

Kingsley, D.H., Guan, D.S. and Hoover, D.G. (2005) Pressure inactivation of hepatitis A virus in strawberry puree and sliced green onions. *Journal of Food Protection* **68**, 1748-1751.

Kingsley, D.H., Hollinian, D.R., Calci, K.R., Chen, H.Q. and Flick, G.J. (2007) Inactivation of a norovirus by high-pressure processing. *Applied and Environmental Microbiology* **73**, 581-585.

Kingsley, D.H., Vincent, E.M., Meade, G.K., Watson, C.L. and Fan, X.T. (2014) Inactivation of human norovirus using chemical sanitizers. *International Journal of Food Microbiology* **171**, 94-99.

Kitajima, M., Oka, T., Haramoto, E., Phanuwan, C., Takeda, N., Katayama, K. and Katayama, H. (2011) Genetic diversity of genogroup IV noroviruses in wastewater in Japan. *Letters in Applied Microbiology* **52**, 181-184.

Kniel, K.E. (2014) The makings of a good human norovirus surrogate. *Current Opinion in Virology* **4**, 85-90.

Knight, A., Haines, J., Stals, A., Li, D., Uyttendaele, M., Jaykus, L. and Knight, A. (2014) A systematic review of the survival of norovirus in foods and on food contact surfaces: Food Standards Agency Project FS241043.

Knight, A., Li, D., Uyttendaele, M. and Jaykus, L.A. (2013) A critical review of methods for detecting human noroviruses and predicting their infectivity. *Critical Reviews in Microbiology* **39**, 295-309.

Knox, J.W., Tyrrel, S.F., Daccache, A. and Weatherhead, E.K. (2011) A geospatial approach to assessing microbiological water quality risks associated with irrigation abstraction. *Water and Environment Journal* **25**, 282-289.

Knudsen, D.M., Yamamoto, S.A. and Harris, L.J. (2001) Survival of *Salmonella* spp. and *Escherichia coli* O157 : H7 on fresh and frozen strawberries. *Journal of Food Protection* **64**, 1483-1488.

Kokkinos, P., Kozyra, I., Lazic, S., Bouwknegt, M., Rutjes, S., Willems, K., Moloney, R., de Roda Husman, A.M., Kaupke, A., Legaki, E., D'Agostino, M., Cook, N., Rzezutka, A., Petrovic, T. and Vantarakis, A. (2012) Harmonised investigation of the occurrence of human enteric viruses in the leafy green vegetable supply chain in three European countries. *Food and Environmental Virology* **4**, 179-191.

Konowalchuk, J. and Speirs, J.I. (1975) Survival of enteric viruses on fresh fruit. *Journal of Milk and Food Technology* **38**, 598-600.

Koopmans, M. and Duizer, E. (2004) Foodborne viruses: an emerging problem. *International Journal of Food Microbiology* **90**, 23-41.

Koopmans, M., von Bonsdorff, C.H., Vinje, J., de Medici, D. and Monroe, S. (2002) Foodborne viruses. *Fems Microbiology Reviews* **26**, 187-205.

Korsager, B., Hede, S., Boggild, H., Böttiger, B.E. and Molbak, K. (2005) Two outbreaks of norovirus infections associated with the consumption of imported frozen raspberries, Denmark, May-June 2005. *Euro Surveillance*, **10**. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=2729.

Kosa, K.M., Cates, S.C., Hall, A.J., Brophy, J.E. and Fraser, A. (2014) Gaps in food safety professionals' knowledge about noroviruses. *Journal of Food Protection* **77**, 6.

Kothary,M.H. and Babu,U.S. (2001) Infective dose of foodborne pathogens in volunteers: A review. *Journal of Food Safety* **21**, 49-73.

Kotwal, G. and Cannon, J.L. (2014) Environmental persistence and transfer of enteric viruses. *Current Opinion in Virology* **4**, 37-43.

Kovac, K., Diez-Valcarce, M., Hernandez, M., Raspor, P. and Rodriguez-Lazaro, D. (2010) High hydrostatic pressure as emergent technology for the elimination of foodborne viruses. *Trends in Food Science & Technology* **21**, 558-568.

Kovac, K., Diez-Valcarce, M., Raspor, P., Hernandez, M. and Rodriguez-Lazaro, D. (2012) Effect of high hydrostatic pressure processing on norovirus infectivity and genome stability in strawberry puree and mineral water. *International Journal of Food Microbiology* **152**, 35-39.

Kozyra, I., Kaupke, A. and Rzezutka, A. (2011) Seasonal occurrence of human enteric viruses in river water samples collected from rural areas of south-east Poland. *Food and Environmental Virology* **3**, 115-120.

Kroes, R., Müller, D., Lambe, J., Löwik, M; R. H., van Klaveren, J., Kleiner, J., Massey, R., Mayer, S., Urieta, I., Vergerj, P. and Visconti, A. (2002) Assessment of intake from the diet. *Food and Chemical Toxicology* **40**, 327-385.

Kroneman, A., Vega, E., Vennema, H., Vinjé, J., White, P. A., Hansman, G., Green, K., Martella, V., Katayama, K. and Koopmans, M. (2013) Proposal for a unified norovirus nomenclature and genotyping. *Archives of Virology* **158**, 2059-2068.

Kurdziel, A.S., Wilkinson, N., Langton, S. and Cook, N. (2001) Survival of poliovirus on soft fruit and salad vegetables. *Journal of Food Protection* **64**, 706-709.

Kuusi, M., Nuorti, J.P., Maunula, L., Minh, N.N.T., Ratia, M., Karlsson, J. and von Bonsdorff, C.H. (2002) A prolonged outbreak of Norwalk-like calicivirus (NLV) gastroenteritis in a rehabilitation centre due to environmental contamination. *Epidemiology and Infection* **129**, 133-138.

L'Homme, Y., Sansregret, R., Plante-Fortier, E., Lamontagne, A.M., Ouardani, M., Lacroix, G. and Simard, C. (2009) Genomic characterization of swine caliciviruses representing a new genus of Caliciviridae. *Virus Genes* **39**, 66-75.

La Rosa, G., Fratini, M., Vennarucci, V.S., Guercio, A., Purpari, G. and Muscillo, M. (2012) GIV noroviruses and other enteric viruses in bivalves: a preliminary study. *New Microbiologica* **35**, 27-34.

La Rosa, G., Iaconelli, M., Pourshaban, M., Fratini, M. and Muscillo, M. (2010) Molecular detection and genetic diversity of norovirus genogroup IV: a yearlong monitoring of sewage throughout Italy. *Archives of Virology* **155**, 589-593.

Lahou, E., Jacxsens, L., Van Landeghem, F. and Uyttendaele, M. (2014) Microbiological sampling plan based on risk classification to verify supplier selection and production of served meals in food service operation. *Food Microbiology* **41**, 60-75.

Lambertini, E., Spencer, S.K., Bertz, P.D., Loge, F.J., Kieke, B.A. and Borchardt, M.A. (2008) Concentration of enteroviruses, adenoviruses, and noroviruses from drinking water by use of glass wool filters. *Applied and Environmental Microbiology* **74**, 2990-2996.

Landry, E.F., Vaughn, J.M., Thomas, M.Z. and Beckwith, C.A. (1979) Adsorption of enteroviruses to soil cores and their subsequent elution by artificial rainwater. *Applied and Environmental Microbiology* **38**, 680-687.

Langlet, J., Gaboriaud, F. and Gantzer, C. (2007) Effects of pH on plaque forming unit counts aggregation of Ms2 bacteriophage. *Journal of Applied Microbiology* **103**, 1632-1638.

Lawrence, L., Kerrod, E., Gani, R. and Leach, S. (2004) Final scientific report: Microbiological risk assessment for norovirus infection - contribution to the overall burden afforded by foodborne infections: Health Protection Agency, Porton Down, UK.

Le Guyader, F.S. and Atmar, R.L. (2008) Binding and inactivation of viruses on and in food, with a focus on the role of the matrix. In *Food-borne viruses: progress and challenges* eds. Koopmans, M.P.G., Cliver, D.O. and Bosch, A. pp.189-208. Washington: ASM Press.

Le Guyader, F.S., Mittelholzer, C., Haugarreau, L., Hedlund, K.O., Alsterlund, R., Pommepuy, M. and Svensson, L. (2004) Detection of noroviruses in raspberries associated with a gastroenteritis outbreak. *International Journal of Food Microbiology* **97**, 179-186.

Le Donne, C., Piccinelli, R., Sette, S., and Leclerq, C. (2011) Overview of existing European food consumption databases: critical aspects in relation to their use for the assessment of dietary exposure to additives, flavouring and residues of food contact materials. *International Journal of Food Science and Nutrition* **62**, 121-132.

Lechevallier, M. and Buckley, M. (2007) Clean water - What is acceptable microbial risk? Washington, DC: Amercian Academy of Microbiology.

Lee, D.Y., Lee, H., Trevors, J.T., Weir, S.C., Thomas, J.L., Habash, M. (2014) Characterization of sources and loadings of fecal pollutants using microbial source tracking assays in urban and rural areas of the Grand River Watershed, Southwestern Ontario. *Water Research* **53**, 123-131.

Lee, G.C., Kim, M.J., Kim, J.I. and Lee, C.H. (2014) Occurrence and molecular characterization of noroviruses in Korean surface water between 2007 and 2010. *Journal of Microbiology and Biotechnology* **24**, 556-562.

Lee, H., Kim, M., Paik, S.Y., Lee, C.H., Jheong, W.H., Kim, J. and Ko, G. (2011) Evaluation of electropositive filtration for recovering norovirus in water. *Journal of Water and Health* **9**, 27-36.

Lee, R.M., Lessler, J., Lee, R.A., Rudolph, K.E., Reich, N.G., Perl, T.M. and Cummings, D.A.T. (2013) Incubation periods of viral gastroenteritis: a systematic review. *Bmc Infectious Diseases* **13**.

Lees, D. (2010) International standardisation of a method for detection of human pathogenic viruses in molluscan shellfish. *Food and Environmental Virology* **2**, 146-155.

LeggeÄ, M. (1997) Watch out when heptatitis A's about. In *The Prague Post - The Czech Repbulic's English-language newspaper.*

Lehto, M., Kuisma, R., Maatta, J., Kymalainen, H.R. and Maki, M. (2011) Hygienic level and surface contamination in fresh-cut vegetable production plants. *Food Control* **22**, 469-475.

Leifert, C., Ball, K., Volakakis, N., Cooper, J. M. (2008) Control of enteric pathogens in ready-to-eat vegetable crops in organic and 'low input' production systems: a HACCP-based approach. *Journal of Applied Microbiology* **105**, 931-950.

Leimbach, A., Hacker, J., and Dobrindt, U. (2013) *E. coli* as an All-Rounder: The thin line between commensalism and pathogenicity. In Between Pathogenicity and Commensalism eds. Dobrindt, U., Hacker, J. H. and Svanborg, C. pp. 3-32. Berlin: Springer-Verlag Berlin.

Leon-Felix, J., Martinez-Bustillos, R.A., Baez-Sanudo, M., Peraza-Garay, F. and Chaidez, C. (2010) Norovirus contamination of bell pepper from handling during harvesting and packing. *Food and Environmental Virology* **2**, 211-217.

Leon, J.S., Kingsley, D.H., Montes, J.S., Richards, G.P., Lyon, G.M., Abdulhafid, G.M., Seitz, S.R., Fernandez, M.L., Teunis, P.F., Flick, G.J. and Moe, C.L. (2011) Randomized, double-blinded clinical trial for human norovirus inactivation in oysters by high hydrostatic pressure processing. *Applied and Environmental Microbiology* **77**, 5476-5482.

Levasseur, V., Pasquini, M.W., Kouame, C. and Temple, L. (2007) A review of urban and peri-urban vegetable production in West Africa. In *Proceedings of the International Symposium on Horticultural Plants in Urban and Peri-Urban Life* eds. Lumpkin, T.A. and Warrington, I.J. pp.245-252. Leuven 1: International Society Horticultural Science.

Lewis, G.D., Molloy, S.L., Greening, G.E. and Dawson, J. (2000) Influence of environmental factors on virus detetion by RT-PCR and cell culture. *Journal of Applied Microbiology* **88**, 633-640.

Lewis, W.J., Foster, S.S.D. and Drasar, B.S. (1980) The risk of groundwater pollution by on-site sanitation in developing countries - A literature review: International Reference Centre for Wastes Disposal (IRCWD).

Li, D. (2012) Use of surrogates and viral binding properties to predict and control human norovirus infectivity. In *PhD dissertation, Faculty of Bioscience Engineering*. Ghent: Ghent University.

Li, D., Baert, L., De Jonghe, M., Van Coillie, E., Ryckeboer, J., Devlieghere, F. and Uyttendaele, M. (2011) Inactivation of murine norovirus 1, coliphage phi X174, and Bacillus fragilispPhage B40-8 on surfaces and fresh-cut iceberg lettuce by hydrogenp peroxide and UV light. *Applied and Environmental Microbiology* **77**, 1399-1404.

Li, D., Baert, L., Zhang, D.S., Xia, M., Zhong, W.M., Van Coillie, E., Jiang, X. and Uyttendaele, M. (2012) Effect of grape seed extract on human norovirus GII. 4 and murine norovirus 1 in viral suspensions, on stainless steel discs, and in lettuce wash water. *Applied and Environmental Microbiology* **78**, 7572-7578.

Li, D., De Keuckelaere, A. and Uyttendaele, M. (2014a) Application of long-range and binding reverse transcription-quantitative PCR to indicate the viral integrities of noroviruses. *Applied and Environmental Microbiology* **80**, 6473-6479.

Li, D., Shi, H.C. and Jiang, S.C. (2010) Concentration of viruses from environmental waters using nanoalumina fiber filters. *Journal of Microbiological Methods* **81**, 33-38.

Li, D., Stals, A., Tang, Q.J. and Uyttendaele, M. (2014b) Detection of noroviruses in shellfish and semiprocessed fishery products from a Belgian seafood company. *Journal of Food Protection* **77**, 1342-1347.

Li, X.H., Chen, H.Q. and Kingsley, D.H. (2013a) The influence of temperature, pH, and water immersion on the high hydrostatic pressure inactivation of GI.1 and GII.4 human noroviruses. *International Journal of Food Microbiology* **167**, 138-143.

Li, X.H., Ye, M., Neetoo, H., Golovan, S. and Chen, H.Q. (2013b) Pressure inactivation of Tulane virus, a candidate surrogate for human norovirus and its potential application in food industry. *International Journal of Food Microbiology* **162**, 37-42.

Lim,K.Y. and Jiang,S.C. (2013) Reevaluation of health risk benchmark for sustainable water practice through risk analysis of rooftop-harvested rainwater. *Water Research* **47**, 7273-7286.

Liu, P., Escudero, B., Jaykus, L-A., Montes, J., Goulter, R., Lichtenstein, M., Fernandez, M., Lee, J-C., De Nardo, E., Kirby, A., Arbogast, J. W. and Moe, C. L. (2013) *Applied and Environmental Microbiology* **79**, 7875-7881.

Liu,C., Hofstra,N., and Franz,E. (2013) Impacts of climate change on the microbial safety of pre-harvest leafy green vegetables as indicated by Escherichia coli O157 and Salmonella spp. *International Journal of Food Microbiology* **163**, 119-128.

Lodder, W.J., van den Berg, H.H.J.L., Rutjes, S.A. and Husman, A.M.D. (2010) Presence of enteric viruses in source waters for drinking water production in the Netherlands. *Applied and Environmental Microbiology* **76**, 5965-5971.

Lopez-Galvez, F., Allende, A., Selma, M.V. and Gil, M.I. (2009) Prevention of Escherichia coli cross-contamination by different commercial sanitizers during washing of fresh-cut lettuce. *International Journal of Food Microbiology* **133**, 167-171.

Lopez-Galvez, F., Allende, A., Truchado, P., Martinez-Sanchez, A., Tudela, J.A., Selma, M.V. and Gil, M.I. (2010a) Suitability of aqueous chlorine dioxide versus sodium hypochlorite as an effective sanitizer for preserving quality of fresh-cut lettuce while avoiding by-product formation. *Postharvest Biology and Technology* **55**, 53-60.

Lopez-Galvez, F., Gil, M.I., Truchado, P., Selma, M.V. and Allende, A. (2010b) Cross-contamination of fresh-cut lettuce after a short-term exposure during pre-washing cannot be controlled after subsequent washing with chlorine dioxide or sodium hypochlorite. *Food Microbiology* **27**, 199-204.

Lopman, B.A., Adak, G.K., Reacher, M.H. and Brown, D.W.G. (2003) Two epidemiologic patterns of Norovirus outbreaks: Surveillance in England and Wales, 1992-2000. *Emerging Infectious Diseases* **9**, 71-77.

Lou, F.F., Neetoo, H., Chen, H.Q. and Li, J.R. (2011) Inactivation of a human norovirus surrogate by high-pressure processing: effectiveness, mechanism, and potential application in the fresh produce industry. *Applied and Environmental Microbiology* **77**, 1862-1871.

Loutreul, J., Cazeaux, C., Levert, D., Nicolas, A., Vautier, S., Le Sauvage, A.L., Perelle, S. and Morin, T. (2014) Prevalence of human noroviruses in frozen marketed shellfish, red fruits and fresh vegetables. *Food Environmental Virology* **6**, 157-168.

Lowther, J.A., Avant, J.M., Gizynski, K., Rangdale, R.E. and Lees, D.N. (2010) Comparison between quantitative real-time reverse transcription PCR results for norovirus in oysters and self-reported gastroenteric illness in restaurant customers. *Journal of Food Protection* **73**, 305-311.

Lowther, J.A., Gustar, N.E., Hartnell, R.E. and Lees, D.N. (2012) Comparison of norovirus RNA levels in outbreak-related oysters with background environmental levels. *Journal of Food Protection* **75**, 389-393.

Lukasik, J., Bradley, M.L., Scott, T.M., Dea, M., Koo, A., Hsu, W.Y., Bartz, J.A. and Farrah, S.R. (2003) Reduction of poliovirus 1, bacteriophages, *Salmonella Montevideo*, and *Escherichia coli* O157 : H7 on strawberries by physical and disinfectant washes. *Journal of Food Protection* **66**, 188-193.

Lukasik, J., Scott, T.M., Andryshak, D. and Farrah, S.R. (2000) Influence of salts on virus adsorption to microporous filters. *Applied and Environmental Microbiology* **66**, 2914-2920.

Luo, Y.G. (2007) Fresh-cut produce wash water reuse affects water quality and packaged product quality and microbial growth in Romaine lettuce. *Hortscience* **42**, 1413-1419.

Luo, Y.G., Nou, X.W., Yang, Y., Alegre, I., Turner, E., Feng, H., Abadias, M. and Conway, W. (2011) Determination of free chlorine concentrations needed to prevent *Escherichia coli* O157:H7 cross-contamination during fresh-cut produce wash. *Journal of Food Protection* **74**, 352-358.

Lutz, J.K., Crawford, J., Hoet, A.E., Wilkins, J.R. and Lee, J. (2013) Comparative performance of contact plates, electrostatic wipes, swabs and a novel sampling device for the detection of Staphylococcus aureus on environmental surfaces. *Journal of Applied Microbiology* **115**, 171-178.

Ma, H.J., Fu, L.L. and Li, J.R. (2011) Differentiation of fecal Escherichia coli from human, livestock, and poultry sources by rep-PCR DNA fingerprinting on the shellfish culture area of East China Sea. *Current Microbiology* **62**, 1423-1430.

Macarisin,D., Patel,J., Bauchan,G., Giron,J.A., and Ravishankar,S. (2013) Effect of spinach cultivar and bacterial adherence factors on survival of *Escherichia coli* O157:H7 on spinach leaves. *Journal of Food Protection* **76**, 1829-1837.

MacDonald, E., Heier, B.T., Stalheim, T., Cudjoe, K.S., Skjerdal, T., Wester, A., Lindstedt, B.A. and Vold, L. (2011) *Yersinia enterocolitica* O:9 infections associated with bagged salad mix in Norway, February to April 2011. *Eurosurveillance* **16**, 10-12.

Mäde, D., Trübner, K., Neubert, E., Höhne, M. and Johne, R. (2013) Detection and typing of norovirus from frozen strawberries involved in a large-scale gastroenteritis outbreak in Germany. *Food and Environmental Virology* **5**, 162-168.

Maimon,A., Tal,A., Friedler,E., and Gross,A. (2010) Safe on-site reuse of greywater for irrigation - A critical review of current guidelines. *Environmental Science & Technology* **44**, 3213-3220.

Makary, P., Maunula, L., Niskanen, T., Kuusi, M., Virtanen, M., Pajunen, S., Ollgren, J. and Minh, N.N.T. (2009) Multiple norovirus outbreaks among workplace canteen users in Finland, July 2006. *Epidemiology and Infection* **137**, 402-407.

Mans, J., Netshikweta, R., Magwalivha, M., Van Zyl, W.B. and Taylor, M.B. (2013) Diverse norovirus genotypes identified in sewage-polluted river water in South Africa. *Epidemiology and Infection* **141**, 303-313.

Mans, J., van Zyl, W.B., Taylor, M.B., Page, N.A., Sobsey, M.D., Barnard, T.G. and Potgieter, N. (2014) Applicability of bio-wipes for the collection of human faecal specimens for detection and characterization of enteric viruses. *Tropical Medicine and international Health* **19**, 293-300.

Manso, C.F. and Romalde, J.L. (2013) Detection and characterization of hepatitis A virus and norovirus in mussels from galicia (NW Spain). *Food and Environmental Virology* **5**, 110-118.

Mansur, A. R., Wang, J., Park, M.-S., and Oh, D.-H. (2014) Growth model of *Escherichia coli* O157:H7 at various storage temperatures on kale treated by thermosonication combined with slightly acidic electrolyzed water. *Journal of Food Protection* **77**, 23-31.

Mara,D. (2011) Water- and wastewater-related disease and infection risks: what is an appropriate value for the maximum tolerable additional burden of disease? *Journal of Water and Health* **9**, 217-224.

Mara, D., Hamilton, A., Sleigh, A., and Karavarsamis, N. (2010) Discussion paper: options for updating the 2006 WHO guidelines. WHO-FAO-IDRC-IWMI.

Mara,D. and Sleigh,A. (2010a) Estimation of *Ascaris* infection risks in children under 15 from the consumption of wastewater-irrigated carrots. *Journal of Water and Health* **8**, 35-38.

Mara,D. and Sleigh,A. (2010b) Estimation of norovirus infection risks to consumers of wastewater-irrigated food crops eaten raw. *Journal of Water and Health* **8**, 39-43.

Mara,D.D., Sleigh,P.A., Blumenthal,U.J., and Carr,R.M. (2007) Health risks in wastewater irrigation: Comparing estimates from quantitative microbial risk analyses and epidemiological studies. *Journal of Water and Health* **5**, 39-50.

Martella, V., Campolo, M., Lorusso, E., Cavicchio, P., Camero, M., Bellacicco, A.L., Decaro, N., Elia, G., Greco, G., Corrente, M., Desario, C., Arista, S., Banyai, K., Koopmans, M. and Buonavoglia, C. (2007) Norovirus in captive lion cub (Panthera leo). *Emerging Infectious Diseases* **13**, 1071-1073.

Martella, V., Lorusso, E., Decaro, N., Elia, G., Radogna, A., D'Abramo, M., Desario, C., Cavalli, A., Corrente, M., Camero, M., Germinario, C.A., Banyai, K., Di Martino, B., Marsilio, F., Carmichael, L.E. and Buonavoglia, C. (2008) Detection and molecular characterization of a canine norovirus. *Emerging Infectious Diseases* **14**, 1306-1308.

Martinez-Abad, A., Ocio, M.J., Lagaron, J.M. and Sanchez, G. (2013) Evaluation of silver-infused polylactide films for inactivation of *Salmonella* and feline calicivirus in vitro and on fresh-cut vegetables. *International Journal of Food Microbiology* **162**, 89-94.

Mathijs, E., Stals, A., Baert, L., Botteldoorn, N., Denayer, S., Mauroy, A., Scipioni, A., Daube, G., Dierick, K., Herman, L., Van Coillie, E., Uyttendaele, M. and Thiry, E. (2012) A review of known and hypothetical transmission routes for noroviruses. *Food and Environmental Virology* **4**, 131-152.

Matthews, J.E., Dickey, B.W., Miller, R.D., Felzer, J.R., Dawson, B.P., Lee, A.S., Rocks, J.J., Kiel, J., Montes, J.S., Moe, C.L., Eisenberg, J.N.S. and Leon, J.S. (2012) The epidemiology of published norovirus outbreaks: a review of risk factors associated with attack rate and genogroup. *Epidemiology and Infection* **140**, 1161-1172.

Mattison, K. and Bidawid, S. (2009) Analytical Methods for Food and Environmental Viruses. *Food and Environmental Virology* **1**, 107-122.

Mattison, K., Brassard, J., Gagne, M.J., Ward, P., Houde, A., Lessard, L., Simard, C., Shukla, A., Pagotto, F., Jones, T.H. and Trottier, Y.L. (2009) The feline calicivirus as a sample process control for the detection of food and waterborne RNA viruses. *International Journal of Food Microbiology* **132**, 73-77.

Mattison, K., Harlow, J., Morton, V., Cook, A., Pollari, F. and Bidawid, S. (2010) Enteric viruses in ready-to-eat packaged leafy greens. *Emerging Infectious Diseases* **16**, 1815-1817.

Mattison, K., Karthikeyan, K., Abebe, M., Malik, N., Sattar, S.A., Farber, J.M. and Bidawid, S. (2007) Survival of calicivirus in foods and on surfaces: Experiments with feline calicivirus as a surrogate for norovirus. *Journal of Food Protection* **70**, 500-503.

Mauffret, A., Caprais, M.P. and Gourmelon, M. (2012) Relevance of Bacteroidales and F-specific RNA bacteriphages for efficient fecal contamination tracking at the level of a catchment in France. *Applied and Environmental Microbiology* **78**, 5143-5152.

Maunula, L., Kaupke, A., Vasickova, P., S"derberg, K., Kozyra, I., Lazic, S., Van der Poel, W.H.M., Bouwknegt, M., Rutjes, S., Willems, K.A., Moloney, R., D'Agostino, M., de Roda Husman, A.M., von Bonsdorff, C.H., Rzezutka, A., Pavlik, I., Petrovic, T. and Cook, N. (2013) Tracing enteric viruses in the European berry fruit supply chain. *International Journal of Food Microbiology* **167**, 177-185.

Maunula, L., Roivainen, M., Keranen, M., Makela, S., Sodenberg, K., Summa, M., von Bonsdorff, C.H., Lappalainen, M., Korhonen, T., Kuusi, M. and Niskanen, T. (2009) Detection of human norovirus from frozen raspberries in a cluster of gastroenteritis outbreaks. *Eurosurveillance* **14**, 16-18.

Maunula, L., Soderberg, K., Vahtera, H., Vuorilehto, V.P., von Bonsdorff, C.H., Valtari, M., Laakso, T. and Lahti, K. (2012) Presence of human noro- and adenoviruses in river and treated wastewater, a longitudinal study and method comparison. *Journal of Water and Health* **10**, 87-99.

Mayr, C., Strohe, G. and Contzen, M. (2009) Detection of rotavirus in food associated with a gastroenteritis outbreak in a mother and child sanatorium. *International Journal of Food Microbiology* **135**, 179-182.

Mbithi, J.N., Springthorpe, V.S., Boulet, J.R. and Sattar, S.A. (1992) Survival of hepatitis A virus on human hands and its transfer on contact with animate and inanimate surfaces. *Journal of Clinical Microbiology* **30**, 757-763.

Mbithi, J.N., Springthorpe, V.S. and Sattar, S.A. (1993) Comparative in-vivo efficiencies of hand-washing agents against hepatitis A virus (HM-175) and poliovirus type-1 (Sabin). *Applied and Environmental Microbiology* **59**, 3463-3469.

McBride, G.B., Stott, R., Miller, W., Bambic, D. and Wuertz, S. (2013) Discharge-based QMRA for estimation of public health risks from exposure to stormwater-borne pathogens in recreational waters in the United States. *Water Research* **47**, 5282-5297.

Melloul, A. A., Hassani, L., and Rafouk, L. (20001) *Salmonella* contamination of vegetables irrigated with untreated wastewater. *World Journal of Microbiology & Biotechnology* **17**, 207-209.

Meltzer, M.I., Shapiro, C.N., Mast, E.E. and Arcari, C. (2001) The economics of vaccinating restaurant workers against hepatitis A. *Vaccine* **19**, 2138-2145.

Menon, V.K., George, S., Aladin, F., Nawaz, S., Sarkar, R., Lopman, B., Gray, J.J., Gomara, M.I. and Kang, G. (2013a) Comparison of age-stratified seroprevalence of antibodies against norovirus GII in India and the United Kingdom. *Plos One* **8**.

Menon, V.K., George, S., Shanti, A.A., Saravanabavan, A., Samuel, P., Ramani, S., Estes, M.K. and Kang, G. (2013b) Exposure to human and bovine noroviruses in a birth cohort in southern India from 2002 to 2006. *Journal of Clinical Microbiology* **51**, 2391-2395.

Menzel, L. and Matovelle, A. (2010) Current state and future development of blue water availability and blue water demand: A view at seven case studies. *Journal of Hydrology* **384**, 245-263.

Mesquita, J.R., Barclay, L., Nascimento, M.S.J. and Vinje, J. (2010) Novel norovirus in dogs with diarrhea. *Emerging Infectious Diseases* **16**, 980-982.

Mesquita, J.R. and Nascimento, M.S.J. (2009) A foodborne outbreak of norovirus gastroenteritis associated with a christmas dinner in Porto, Portugal, December 2008. *Eurosurveillance* **14**, 19-21.

Mesquita, J.R. and Nascimento, M.S.J. (2014) Norovirus GII.4 antibodies in the Portuguese population. *Journal of Infection in Developing Countries* **8**, 1201-1204.

Miagostovich, M.P., Ferreira, F.F.M., Guimaraes, F.R., Fumian, T.M., Diniz-Mendes, L., Luz, S.L.B., Silva, L.A. and Leite, J.P.G. (2008) Molecular detection and characterization of gastroenteritis viruses occurring naturally in the stream waters of manaus, Central Amazonia, Brazil. *Applied and Environmental Microbiology* **74**, 375-382.

Michen, B. and Graule, T. (2010) Isoelectric points of viruses. *Journal of Applied Microbiology* **109**, 388-397.

Mieszkin, S., Caprais, M.P., Le Mennec, C., Le Goff, M., Edge, T.A. and Gourmelon, M. (2013) Identification of the origin of faecal contamination in estuarine oysters using Bacteroidales and F-specific RNA bacteriophage markers. *Journal of Applied Microbiology* **115**, 897-907.

Mok, H. F., Barker, S. F., and Hamilton, A. J. (2014) A probabilistic quantitative microbial risk assessment model of norovirus disease burden from wastewater irrigation of vegetables in Shepparton, Australia. *Water Research* **54**, 347-362.

Mokhtari, A. and Jaykus, L.A. (2009) Quantitative exposure model for the transmission of norovirus in retail food preparation. *International Journal of Food Microbiology* **133**, 38-47.

Moore, G. and Griffith, C. (2002) A comparison of traditional and recently developed methods for monitoring surface hygiene within the food industry: an industry trial. *International Journal of Environmental Health Research* **12**, 317-329.

Moore, G. and Griffith, C. (2007) Problems associated with traditional hygiene swabbing: the need for in-house standardization. *Journal of Applied Microbiology* **103**, 1090-1103.

Morgenthaler, J.J. (2001) Securing viral safety for plasma derivatives. *Transfusion Medicine Reviews* **15**, 224-233.

Mossel, D.A.A., Corry, J.E.L., Struijk, C.B. and Baird, R.M. (1995) Essentials of the microbiology of foods: A textbook for advanced studies. West Sussex, England: John Wiley & Sons, 699 pp.

Mota,A., Mena,K.D., Soto-Beltran,M., Tarwater,P.M., and Chaidez,C. (2009) Risk assessment of *Cryptosporidium* and *Giardia* in water irrigating fresh produce in Mexico. *Journal of Food Protection* **72**, 2184-2188.

Mukherjee, A., Speh, D., Jones, A.T., Buesing, K.M. and Diez-Gonzalez, F. (2006) Longitudinal microbiological survey of fresh produce grown by farmers in the upper midwest. *Journal of Food Protection* **69**, 1928-1936.

Muniain-Mujika, I., Calvo, M., Lucena, F. and Girones, R. (2003) Comparative analysis of viral pathogens and potential indicators in shellfish. *International Journal of Food Microbiology* **83**, 75-85.

Munoz,I., Tomas,N., Mas,J., Garcia-Reyes,J.F., Molina-Diaz,A., and Fernandez-Alba,A.R. (2010) Potential chemical and microbiological risks on human health from urban wastewater reuse in

agriculture. Case study of wastewater effluents in Spain. *Journal of Environmental Science and Health Part B-Pesticides Food Contaminants and Agricultural Wastes* **45**, 300-309.

Muscillo, M., Fratini, M., Graffeo, R., Sanguinetti, M., Martella, V., Green, K.Y., Della Libera, S. and La Rosa, G. (2013) GIV Noroviruses in wastewaters and in stool specimens from hospitalized patients. *Food and Environmental Virology* **5**, 194-202.

Nakanishi, K., Tsugawa, T., Honma, S., Nakata, S., Tatsumi, M., Yoto, Y. and Tsutsumi, H. (2009) Detection of enteric viruses in rectal swabs from children with acute gastroenteritis attending the pediatric outpatient clinics in Sapporo, Japan. *Journal of Clinical Virology* **46**, 94-97.

Nasser, A.M. and Metcalf, T.G. (1987) Production of cytopathology in Frhk-4 cells by Bs-C-1-passaged hepatitis a-virus. *Applied and Environmental Microbiology* **53**, 2967-2971.

Navarro, I. and Jimenez, B. (2011) Evaluation of the WHO helminth eggs criteria using a QMRA approach for the safe reuse of wastewater and sludge in developing countries. *Water Science and Technology* **63**, 1499-1505.

Navarro,I., Jimenez,B., Cifuentes,E., and Lucario,S. (2009) Application of Helminth ova infection dose curve to estimate the risks associated with biosolid application on soil. *Journal of Water and Health* **7**, 31-44.

Niemira, B.A. (2003) Irradiation of fresh and minimally processed fruits, vegetables, and juices. In *The microbial safty of minimally processed foods* eds. Novak, J.S., Sapers, G.M. and Juneja, V.K. pp.279-300. Boca Raton, FL: CRC Press.

Niu, M.T., Polish, L.B., Robertson, B.H., Khanna, B.K., Woodruff, B.A., Shapiro, C.N., Miller, M.A., Smith, J.D., Gedrose, J.K., Alter, M.J. and Margolis, H.S. (1992) Multistate outbreak of hepatitis A associated with frozen strawberries. *Journal of Infectious Diseases* **166**, 518-524.

Nnane, D.E., Ebdon, J.E., and Taylor, H.D. (2011) Integrated analysis of water quality parameters for cost-effective faecal pollution management in river catchments. *Water Research* **45**, 2235-2246.

Nordgren, J., Kindberg, E., Lindgren, P.E., Matussek, A. and Svensson, L. (2010) Norovirus gastroenteritis outbreak with a secretor-independent susceptibility pattern, Sweden. *Emerging Infectious Diseases* **16**, 81-87.

Nordgren, J., Nitiema, L.W., Ouermi, D., Simpore, J. and Svensson, L. (2013) Host genetic factors affect susceptibility to norovirus infections in Burkina Faso. *Plos One* **8**.

Nordic outbreak investigation, t. (2013) Joint analysis by the Nordic countries of hepatitis A outbreak, October 2012 to June 2013: frozen strawberries suspected. *Euro Surveillance* **18**.

Normalisation, A.f.d. (2004) RAPID'E. coli 2 validated for the enumeration of coliforms at 37°C. N° BRD-07/0S-12/04. *Association française de Normalisation*.

Nowak, P., Topping, J.R., Bellamy, K., Fotheringham, V., Gray, J.J., Golding, J.P., Wiseman, G., Knight, A.I. (2011) Virolysis of feline calicivirus and human GII.4 norovirus following chlorine exposre under tsandardized light soil disinfection conditions. *Journal of Food Protection* **74**, 2113-2118.

NRMMC-EPHC-AHMC (Natural Resource Management Ministerial Council, Environment Protection and Hertigae Council, Australia Health Ministers' Conference) (2006) Australian guidelines for water

recycling: managing health and environmental risks: Phase 1. National water quality management strategy.NRMMC-EPHC-NHMRC, Canberra, Australia, -389.

Nurminen, K., Blazevic, V., Huhti, L., Rasanen, S., Koho, T., Hytonen, V.P. and Vesikari, T. (2011) Prevalence of norovirus GII-4 antibodies in Finnish children. *Journal of Medical Virology* **83**, 525-531.

Nygard, K., Andersson, Y., Lindkvist, P., Ancker, C., Asteberg, I., Dannetun, E., Eitrem, R., Hellstrom, L., Insulander, M., Skedebrant, L., Stenqvist, K. and Giesecke, J.G. (2001) Imported rocket salad partly responsible for increased incidence of hepatitis A cases in Sweden. *Euro Surveillance* **6**, 151-153.

O'Toole, J., Sinclair, M., Barker, S. F., and Leder, K. (2014) Advice to risk assessors modeling viral health risk associated with household graywater. *Risk Analysis* **34**, 797-802.

O'Toole, J., Sinclair, M., and Leder, K. (2010) Quantitative microbial risk assessment and Australian Guidelines for Water Recycling: two case studies. *Food Australia* **62**, 408-412.

O'Toole, J., Sinclair, M., Malawaraarachchi, M., Hamilton, A., Barker, S.F., and Leder, K. (2012) Microbial quality assessment of household greywater. *Water Research* **46**, 4301-4313.

Obuobie, E., Keraita, B., Danso, G., Amoah, P., Cofie, O. O., Raschid-Sally, L., and Drechsel, P. (2006) Irrigated urban vegetable production in Ghana: characteristic benefits and risks. IWMI-RUAF-CFWF, Accra, Ghana. Available online: www.ruaforg/node/1046

Oishi, I., Yamazaki, K., Kimoto, T., Minekawa, Y., Utagawa, E., Yamazaki, S., Inouye, S., Grohmann, G.S., Monroe, S.S., Stine, S.E., Carcamo, C., Ando, T. and Glass, R.I. (1994) A large outbreak of acute gastroenteritis associated with astrovirus among students and teachers in Osaka, Japan. *Journal of Infectious Diseases* **170**, 439-443.

Okabayashi, T., Yokota, S., Ohkoshi, Y., Ohuchi, H., Yoshida, Y., Kikuchi, M., Yano, K. and Fujii, N. (2008) Occurrence of norovirus infections unrelated to norovirus outbreaks in an asymptomatic food handler population. *Journal of Clinical Microbiology* **46**, 1985-1988.

Okafo, C. N., Umoh, V. J., and Galadima, M.(2003) Occurrence of pathogens on vegetables harvested from soils irrigated with contaminated streams. *Science of the Total Environment* **311**, 49-56.

Olaimat, A. N. and Holley, R. A. (2012) Factors influencing the microbial safety of fresh produce: A review. *Food Microbiology* **32**, 1-19.

Oliver, S.L., Asobayire, E., Dastjerdi, A.M. and Bridger, J.C. (2006) Genomic characterization of the unclassified bovine enteric virus Newbury agent-1 (Newbury1) endorses a new genus in the family Caliciviridae. *Virology* **350**, 240-250.

Oliver, S.L., Dastjerdi, A.M., Wong, S., El-Attar, L., Gallimore, C., Brown, D.W.G., Green, J. and Bridger, J.C. (2003) Molecular characterization of bovine enteric caliciviruses: a distinct third genogroup of noroviruses (Norwalk-like viruses) unlikely to be of risk to humans. *Journal of Virology* **77**, 2789-2798.

Olmez, H. and Kretzschmar, U. (2009) Potential alternative disinfection methods for organic fresh-cut industry for minimizing water consumption and environmental impact. *Lwt-Food Science and Technology* **42**, 686-693.

Olszewski, J., Winona, L. and Oshima, K.H. (2005) Comparison of 2 ultrafiltration systems for the concentration of seeded viruses from environmental waters. *Canadian Journal of Microbiology* **51**, 295-303.

Ongeng, D., Vasquez, G.A., Muyanja, C., Ryckeboer, J., Geeraerd, A.H. and Springael, D. (2011) Transfer and internalisation of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in cabbage cultivated on contaminated manure-amended soil under tropical field conditions in Sub-Saharan Africa. *International Journal of Food Microbiology* **145**, 301-310.

Oogane, F., Hirata, A., Funatogawa, K., Kobayashi, K., Sato, T. and Kimura, H. (2008) Food poisoning outbreak caused by norovirus GII/4 in school lunch, Tochigi Prefecture, Japan. *Japanese Journal of Infectious Diseases* **61**, 423-424.

Oragui, J.I., Curtis, T.P., Silva, S.A., and Mara, D.D. (1987) The removal of excreted bacteria and viruses in deep waste stabilization ponds in northeast Brazil. *Water Science and Technology* **19**, 569-573.

Oron, G., Gillerman, L., Lael, A., Manor, Y., Braude, E., and Bick, A. (2010) Minimizing health risks during secondary effluent application via subsurface drip irrigation. *Water Science and Technology* **62**, 2330-2337.

Ottoson, J., Hansen, A., Westrell, T., Johansen, K., Norder, H., and Stenstrom, T.A. (2006) Removal of noro- and enteroviruses, Giardia cysts, Cryptosporidium oocysts, and fecal indicators at four secondary wastewater treatment plants in Sweden. *Water Environment Research* **78**, 828-834.

Ottoson, J.R., Nyberg, K., Lindqvist, R., and Albihn, A. (2011) Quantitative microbial risk assessment for Escherichia coli O157 on lettuce, based on survival data from controlled studies in a climate chamber. *Journal of Food Protection* **74**, 2000-2007.

Ottoson, J. and Stenstrom, T. A. (2003) Faecal contamination of greywater and associated microbial risks. *Water Research* **37**, 645-655.

Ozawa, K., Oka, T., Takeda, N. and Hansman, G.S. (2007) Norovirus infections in symptomatic and asymptomatic food handlers in Japan. *Journal of Clinical Microbiology* **45**, 3996-4005.

Pachepsky, Y., Shelton, D.R., McLain, J.E.T., Patel, J. and Mandrell, R.E. (2011) Irrigation waters as a source of pathogenic microorganisms inpProduce: A Review. *Advances in Agronomy*, **113**, 73-138.

Palumbo, M., Harris, L.J. and Danyluk, M.D. (2013) Outbreaks of foodborne illness associated with common berries, 1983 through May 2013. p.9: Food Science and Human Nutrition Department, UF/IFAS Extension.

Papafragkou, E., D'Souza, D.H. and Jaykus, L.-A. (2006) Food-borne viruses: prevention and control. In *Viruses in Foods* ed. Goyal, S.M. p.345. United States of America: Springer.

Papafragkou, E., Plante, M., Mattison, K., Bidawid, S., Karthikeyan, K., Farber, J.M. and Jaykus, L.A. (2008) Rapid and sensitive detection of hepatitis A virus in representative food matrices. *Journal of Virological Methods* **147**, 177-187.

Parish, E.S., Kodra, E., Steinhaeuser, K. and Ganguly, A.R. (2012) Estimating future global per capita water availability based on changes in climate and population. *Computers & Geosciences* **42**, 79-86.

Park, G.W., Barclay, L., Macinga, D., Charbonneau, D., Pettigrew, C.A. and Vinje, J. (2010a) Comparative efficacy of seven hand sanitizers against murine norovirus, feline calicivirus, and GII.4 norovirus. *Journal of Food Protection* **73**, 2232-2238.

Park, S.H., Kim, E.J., Yun, T.H., Lee, J.H., Kim, C.K., Seo, Y.H., Oh, S.A., Choi, S.S., Cho, S.J., Kim, M.S., Han, G.Y., Kim, M.Y., Jeong, H.S., Cheon, D.S. and Kim, H.S. (2010b) Human enteric viruses in groundwater. *Food and Environmental Virology* **2**, 69-73.

Patel, M.M., Hall, A.J., Vinje, J. and Parashara, U.D. (2009) Noroviruses: A comprehensive review. *Journal of Clinical Virology* **44**, 1-8.

Patel, J., Sharma, M. and Ravishakar, S. (2011) Effect of curli expression and hydrophobicity of Escherichia coli O157:H7 on attachment to fresh produce surfaces. *Journal of Applied Microbiology* **110**, 737-745.

Patterson, W., Haswell, P., Fryers, P.T. and Green, J. (1997) Outbreak of small round structured virus gastroenteritis arose after kitchen assistant vomited. *Communicable disease reportCDR review* **7**, 101-103.

Pavione,D.M.S., Bastos,R.K.X., and Bevilacqua,P.D. (2013) Quantitative microbial risk assessment applied to irrigation of salad crops with waste stabilization pond effluents. *Water Science and Technology* **67**, 1208-1215.

Pebody, R.G., Leino, T., Ruutu, P., Kinnunen, L., Davidkin, I., Nohynek, H. and Leinikki, P. (1998) Foodborne outbreaks of hepatitis A in a low endemic country: an emerging problem? *Epidemiology and Infection* **120**, 55-59.

Pelosi, E., Lambden, P.R., Caul, E.O., Liu, B.L., Dingle, K., Deng, Y. and Clarke, I.N. (1999) The seroepidemiology of genogroup 1 and genogroup 2 Norwalk-like viruses in Italy. *Journal of Medical Virology* **58**, 93-99.

Perez-Rodriguez, F., Gonzalez-Garcia, P., Valero, A., Hernandez, M. and Rodriguez-Lazaro, D. (2014) Impact of the prevalence of different pathogens on the performance of sampling plans in lettuce products. *International Journal of Food Microbiology* **184**, 69-73.

Petrignani, M., Verhoef, L., Vennema, H., van Hunen, R., Baas, D., van Steenbergen, J.E. and Koopmans, M.P.G. (2014) Underdiagnosis of foodborne hepatitis A, the Netherlands, 2008-2010. *Emerging Infectious Diseases* **20**, 596-602.

Petterson,S.R. and Ashbolt,N.J. (2001) Viral risks associated with wastewater reuse: modeling virus persistence on wastewater irrigated salad crops. *Water Science and Technology* **43**, 23-26.

Petterson,S.R., Ashbolt,N.J., and Sharma,A. (2001a) Microbial risks from wastewater irrigation of salad crops: A screening-level risk assessment. *Water Environment Research* **73**, 667-672.

Petterson,S.R., Ashbolt,N.J., and Sharma,A. (2002) Of: microbial risks from wastewater irrigation of salad crops: a screening-level risk assessment. *Water Environment Research* **74**, 411.

Petterson, S.R., Teunis, P.F.M. and Ashbolt, N.J. (2001b) Modeling virus inactivation on salad crops using microbial count data. *Risk Analysis* **21**, 1097-1108.

Phillips, G., Tam, C.C., Rodrigues, L.C. and Lopman, B. (2010) Prevalence and characteristics of asymptomatic norovirus infection in the community in England. *Epidemiology and Infection* **138**, 1454-1458.

Pielaat, A., van Leusden, F., and Wijnands, L. (2014) Microbiological risk from minimally processed packaged salads in the Dutch food chain. *Journal of Food Protection* **77**, 395-403.

Pina, S., Puig, M., Lucena, F., Jofre, J. and Girones, R. (1998) Viral pollution in the environment and in shellfish: human adenovirus detection by PCR as an index of human viruses. *Applied and Environmental Microbiology* **64**, 3376-3382.

Pönkä, A., Maunula, L., von Bonsdorff, C.H. and Lyytikainen, O. (1999a) An outbreak of calicivirus associated with consumption of frozen raspberries. *Epidemiology and Infection* **123**, 469-474.

Pönkä, A., Maunula, L., Von Bonsdorff, C.H. and Lyytikainen, O. (1999b) Outbreak of calicivirus gastroenteritis associated with eating frozen raspberries. *Eurosurveillance* **4**, Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=56

Poovorawan, Y., Chatchatee, P. and Chongsrisawat, V. (2002) Epidemiology and prophylaxis of viral hepatitis: A global perspective. *Journal of Gastroenterology and Hepatology* **17**, S155-S166.

Prado, T., Gaspar, A.M.C. and Miagostovich, M.P. (2014) Detection of enteric viruses in activated sludge by feasible concentration methods. *Brazilian Journal of Microbiology* **45**, 343-349.

Predmore, A. and Li, J.R. (2011) Enhanced removal of a human norovirus surrogate from fresh vegetables and fruits by a combination of surfactants and sanitizers. *Applied and Environmental Microbiology* **77**, 4829-4838.

Puerta-Gomez, A.F., Kim, J., Moreira, R.G., Klutke, G.A., and Castell-Perez, M.E. (2013) Quantitative assessment of the effectiveness of intervention steps to reduce the risk of contamination of ready-to-eat baby spinach with Salmonella. *Food Control* **31**, 410-418.

Ragaert, P., Jacxsens, L., Vandekinderen, I., Baert, L. and Devlieghere, F. (2011) Microbiological and safety aspects of fresh-cut fruits and vegetables. In *Advances in fresh-cut fruits and vegetables processing* eds. Martine-Belloso, O. and Soliva-Fortuny, R. pp.53-86. Florida: Taylor & Francis Group.

Ramani, S., Atmar, R.L. and Estes, M.K. (2014) Epidemiology of human noroviruses and updates on vaccine development. *Current Opinion in Gastroenterology* **30**, 25-33.

Ramsay, C.N. and Upton, P.A. (1989) Hepatitis-A and frozen raspberries. *Lancet* **1**, 43-44.

Reid, T.M.S. and Robinson, H.G. (1987) Frozen raspberries and hepatitis-A. *Epidemiology and Infection* **98**, 109-112.

Reij, M.W., and van Schothorst, M. (2000) Critical notes on microbiological risk assessment of food. *Brazilian Journal of Microbiology* **31**, 1-8.

Repp, K.K., Hostetler, T.P. and Keene, W.E. (2013) A norovirus outbreak related to contaminated surfaces. *Journal of Infectious Diseases* **208**, 295-298.

Richards, G.P. (2012) Critical review of norovirus surrogates in food safety research: Rationale for considering volunteer studies. *Food and Environmental Virology* **4**, 6-13.

Richards, G.P., Watson, M.A. and Kingsley, D.H. (2004) A SYBR green, real-time RT-PCR method to detect and quantitate Norwalk virus in stools. *Journal of Virological Methods* **116**, 63-70.

Richards, G.P., Watson, M.A., Meade, G.K., Hovan, G.L. and Kingsley, D.H. (2012) Resilience of norovirus GII.4 to freezing and thawing: implications for virus infectivity. *Food and Environmental Virology* **4**, 192-197.

Rico, D., Martin-Diana, A.B., Barat, J.M. and Barry-Ryan, C. (2007) Extending and measuring the quality of fresh-cut fruit and vegetables: a review. *Trends in Food Science & Technology* **18**, 373-386.

Rimhanen-Finne,R., Vuorinen,A., Marmo,S., Malmberg,S., and Hanninen,M.L. (2004) Comparative analysis of *Cryptosporidium*, *Giardia* and indicator bacteria during sewage sludge hygienization in various composting processes. *Letters in Applied Microbiology* **38**, 301-305.

Rivm (2013) Efficacy of applied processing measures on virus reduction in food. *RIVM Report 330371007/2013*, 1-36.

Rizzo, C., Alfonsi, V., Bruni, R., Busani, L., Ciccaglione, A.R., de Medici, D., di Pasquale, S., Equestre, M., Escher, M., Montano-Remacha, M.C., Scavia, G., Taffon, S., Carraro, V., Franchini, S., Natter, B., Augschiller, M., Tosti, M.E. and A, t.C.T.F.o.H. (2013) Ongoing outbreak of hepatitis A in Italy: preliminary report as of 31 May 2013. *Euro Surveillance* **18**, 6 pp.

Rodriguez, F.P., Campos, D., Ryser, E.T., Buchholz, A.L., Posada-Izquierdo, G.D., Marks, B.P., Zurera, G., and Todd, E. (2011) A mathematical risk model for *Escherichia coli* O157:H7 cross-contamination of lettuce during processing. *Food Microbiology* **28**, 694-701.

Rodriguez-Manzano, J., Alonso, J.L., Ferrus, M.A., Moreno, Y., Amoros, I., Calgua, B., Hundesa, A., Guerrero-Latorre, L., Carratala, A., Rusinol, M., and Girones, R. (2012) Standard and new faecal indicators and pathogens in sewage treatment plants, microbiological parameters for improving the control of reclaimed water. *Water Science and Technology* **66**, 2517-2523.

Roll, B.M. and Fujioka, R.S. (1997) Sources of faecal indicator bacteria in a brackish, tropical stream and their impact on recreational water quality. *Water Science and Technology* **35**, 179-186.

Rönnqvist, M., Aho, E., Mikkelä, A., Ranta, J., Tuominen, P., Rättö, M. and Maunula, L. (2014) Norovirus transmission between hands, gloves, utensils, and fresh produce during simulated food handling. *Applied and Environmental Microbiology* **80**, 5403-5410.

Rönnqvist, M., Rättö, M., Tuominen, P., Salo, S. and Maunula, L. (2013) Swabs as a tool for monitoring the presence of norovirus on environmental surfaces in the food industry. *Journal of Food Protection* **76**, 1421-1428.

Rosenblum, L.S., Mirkin, I.R., Allen, D.T., Safford, S. and Hadler, S.C. (1990) A multifocal outbreak of hepatitis A traced to commercially distributed lettuce. *American Journal of Public Health* **80**, 1075-1079.

Russell, K.L., Broderick, M.P., Franklin, S.E., Blyn, L.B., Freed, N.E., Moradi, E., Ecker, D.J., Kammerer, P.E., Osuna, M.A., Kajon, A.E., Morn, C.B. and Ryan, M.A.K. (2006) Transmission dynamics and prospective environmental sampling of adenovirus in a military recruit setting. *Journal of Infectious Diseases* **194**, 877-885.

Rzeutka, A., D'Agostino, M. and Cook, N. (2006) An ultracentrifugation-based approach to the detection of hepatitis A virus in soft fruits. *International Journal of Food Microbiology* **108**, 315-320.

Rzezutka, A. and Cook, N. (2004) Survival of human enteric viruses in the environment and food. *Fems Microbiology Reviews* **28**, 441-453.

Sales-Ortells, H., Fernandez-Cassi, X., Timoneda, N., Dürig, W., Girones, R. and Medema, G. (2014) Health risks derived from consumption of lettuces irrigated with tertiary effluent containing norovirus. *Food Research International*, available online: http://dxdoiorg/101016/jfoodres201408018.

Sanchez, A. (2013) Hepatitis A virus in food: Detection and inactivation methods. In *SpringerBriefs in food, health, and nutrition* ed. Hartel, R.W. p.47: Springer.

Sanchez, G., Bosch, A. and Pinto, R.M. (2007) Hepatitis A virus detection in food: current and future prospects. *Letters in Applied Microbiology* **45**, 1-5.

Sanchez, G., Elizaquivel, P. and Aznar, R. (2012) A single method for recovery and concentration of enteric viruses and bacteria from fresh-cut vegetables. *International Journal of Food Microbiology* **152**, 9-13.

Sanglay, G.C., Li, J.R., Uribe, R.M. and Lee, K. (2011) Electron-beam inactivation of a norovirus surrogate in fresh produce and model systems. *Journal of Food Protection* **74**, 1155-1160.

Sarvikivi, E., Roivainen, M., Maunula, L., Niskanen, T., Korhonen, T., Lappalainen, M. and Kuusi, M. (2012) Multiple norovirus outbreaks linked to imported frozen raspberries. *Epidemiology and Infection* **140**, 260-267.

Sattar, S.A., Tetro, J., Bidawid, S. and Farber, J. (2000) Foodborne spread of hepatitis A: recent studies on virus survival, transfer and inactivation. *The Canadian journal of infectious diseases* **11**.

Savichtcheva, O. and Okabe, S. (2006) Alternative indicators of fecal pollution: Relations with pathogens and conventional indicators, current methodologies for direct pathogen monitoring and future application perspectives. *Water Research* **40**, 2463-2476.

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.A., Roy, S.L., Jones, J.L. and Griffin, P.M. (2011) Foodborne illness acquired in the United States - Major pathogens. *Emerging Infectious Diseases* **17**, 7-15.

SCF (2002) Risk profile on the microbiological contamination of fruits and vegetables eaten raw. European commission health & consumer protection directorate-general. Report of the scientific committee on food. online available: http://ec.europa.eu/food/fs/sc/scf/out125_en.pdf

Scherer, K., Johne, R., Schrader, C., Ellerbroek, L., Schulenburg, J. and Klein, G. (2010) Comparison of two extraction methods for viruses in food and application in a norovirus gastroenteritis outbreak. *Journal of Virological Methods* **169**, 22-28.

Scherer, K., Made, D., Ellerbroek, L., Schulenburg, J., Johne, R. and Klein, G. (2009) Application of a swab sampling method for the detection of norovirus and rotavirus on artificially contaminated food and environmental surfaces. *Food and Environmental Virology* **1**, 42-49.

Schlindwein, A.D., Rigotto, C., Simoes, C.M.O. and Barardi, C.R.M. (2010) Detection of enteric viruses in sewage sludge and treated wastewater effluent. *Water Science and Technology* **61**, 537-544.

Schmid, D., Stuger, H.P., Lederer, I., Pichler, A.M., Kainz-Arnfelser, G., Schreier, E. and Allerberger, F. (2007) A foodborne norovirus outbreak due to manually prepared salad, Austria 2006. *Infection* **35**, 232-239.

Schrader, C., Schielke, A., Ellerbroek, L. and Johne, R. (2012) PCR inhibitors - occurrence, properties and removal. *Journal of Applied Microbiology* **113**, 1014-1026.

Scott, T.M., Lukasik, J. and Farrah, S.R. (2002) Improved method for recovery of bacteriophage from large volumes of water using negatively charged microporous filters. *Canadian Journal of Microbiology* **48**, 305-310.

Sedmak, G., Bina, D. and MacDonald, J. (2003) Assessment of an enterovirus sewage surveillance system by comparison of clinical isolates with sewage isolates from Milwaukee, Wisconsin, collected August 1994 to December 2002. *Applied and Environmental Microbiology* **69**, 7181-7187.

Seidu, R., Heistad, A., Amoah, P., Drechsel, P., Jenssen, P.D., and Stenstrom, T.A. (2008) Quantification of the health risk associated with wastewater reuse in Accra, Ghana: a contribution toward local guidelines. *Journal of Water and Health* **6**, 461-471.

Seidu, R., Sjolander, I., Abubakari, A., Amoah, D., Larbi, J.A., and Stenstrom, T.A. (2013) Modeling the die-off of E. coli and Ascaris in wastewater-irrigated vegetables: implications for microbial health risk reduction associated with irrigation cessation. *Water Science and Technology* **68**, 1013-1021.

Seitz, S.R., Leon, J.S., Schwab, K.J., Lyon, G.M., Dowd, M., McDaniels, M., Abdulhafid, G., Fernandez, M.L., Lindesmith, L.C., Baric, R.S. and Moe, C.L. (2011) Norovirus infectivity in humans and persistence in water. *Applied and Environmental Microbiology* **77**, 6884-6888.

Selma, M.V., Allende, A., Lopez-Galvez, F., Conesa, M.A. and Gil, M.I. (2008) Disinfection potential of ozone, ultraviolet-C and their combination in wash water for the fresh-cut vegetable industry. *Food Microbiology* **25**, 809-814.

Seo, K., Lee, J.E., Lim, M.Y. and Ko, G. (2012) Effect of Temperature, pH, and NaCl on the Inactivation Kinetics of Murine Norovirus. *Journal of Food Protection* **75**, 533-540.

Seo, K.H. and Frank, J.F. (1999) Attachment of *Escherichia coli* O157 : H7 to lettuce leaf surface and bacterial viability in response to chlorine treatment as demonstrated by using confocal scanning laser microscopy. *Journal of Food Protection* **62**, 3-9.

Serracca, L., Rossini, I., Battistini, R., Goria, M., Sant, S., De Montis, G. and Ercolini, C. (2012) Potential risk of norovirus infection due to the consumption of "Ready to Eat" food. *Food and Environmental Virology* **4**, 89-92.

Seymour, I.J. and Appleton, H. (2001) Foodborne viruses and fresh produce. *Journal of Applied Microbiology* **91**, 759-773.

Shanker, S., Czako, R., Sankaran, B., Atmar, R.L., Estes, M.K. and Prasad, B.V.V. (2014) Structural analysis of determinants of histo-blood group antigen binding specificity in genogroup I noroviruses. *Journal of Virology* **88**, 6168-6180.

Sharps, C.P., Kotwal, G. and Cannon, J.L. (2012) Human norovirus transfer to stainless steel and small fruits during handling. *Journal of Food Protection* **75**, 1437-1446.

Shaw, R.K., Berger, C.N., Feys, B., Knutton, S., Pallen, M.J. and Frankel, G. (2008) Enterohemorrhagic *Escherichia coli* exploits EspA filaments for attachment to salad leaves. *Applied and Environmental Microbiology* **74**, 2908-2914.

Shieh, Y.C., Stewart, D.S. and Laird, D.T. (2009) Survival of hepatitis A virus in spinach during low temperature storage. *Journal of Food Protection* **72**, 2390-2393.

Shieh, Y.C., Tortorello, M.L., Fleischman, G.J., Li, D. and Schaffner, D.W. (2014) Tracking and modeling norovirus transmission during mechanical slicing of globe tomatoes. *International Journal of Food Microbiology* **180**, 13-18.

Shields, P.A. and Farrah, S.R. (1983) Influence of salts on electrostatic interactions between poliovirus and membrane filters. *Applied and Environmental Microbiology* **45**, 526-531.

Shimasaki, N., Kiyohara, T., Totsuka, A., Nojima, K., Okada, Y., Yamaguchi, K., Kajioka, J., Wakita, T. and Yoneyama, T. (2009) Inactivation of hepatitis A virus by heat and high hydrostatic pressure: variation among laboratory strains. *Vox Sanguinis* **96**, 14-19.

Shinohara, M., Uchida, K., Shimada, S., Tomioka, K., Suzuki, N., Minegishi, T., Kawahashi, S., Yoshikawa, Y. and Ohashi, N. (2013) Application of a simple method using minute particles of amorphous calcium phosphate for recovery of norovirus from cabbage, lettuce, and ham. *Journal of Virological Methods* **187**, 153-158.

Showell, D., Sundkvist, T., Reacher, M. and Gray, J. (2007) Norovirus outbreak associated with canteen salad in Suffolk, United Kingdom. *Euro Surveillance* **12**.

Shuval, H., Lampert, Y., and Fattal, B. (1997) Development of a risk assessment approach for evaluating wastewater reuse standards for agriculture. *Water Science and Technology* **35**, 15-20.

Sidhu, J.P.S. and Toze, S.G. (2009) Human pathogens and their indicators in biosolids: A literature review. *Environment International* **35**, 187-201.

Siebenga, J.J., Vennema, H., Duizer, E. and Koopmans, M.P.G. (2007) Gastroenteritis caused by norovirus GGII.4, the Netherlands, 1994-2005. *Emerging Infectious Diseases* **13**, 144-146.

Silva, H.D., Garcia-Zapata, M.T.A. and Anunciacao, C.E. (2011) Why the use of adenoviruses as water quality virologic marker? *Food and Environmental Virology* **3**, 138-140.

Silverman, A.I., Akrong, M.O., Amoah, P., Drechsel, P., and Nelson, K.L. (2013) Quantification of human norovirus GII, human adenovirus, and fecal indicator organisms in wastewater used for irrigation in Accra, Ghana. *Journal of Water and Health* **11**, 473-488.

Sima, L.C., Schaeffer, J., Le Saux, J.C., Parnaudeau, S., Elimelech, M. and Le Guyader, F.S. (2011) Calicivirus Removal in a Membrane Bioreactor Wastewater Treatment Plant. *Applied and Environmental Microbiology* **77**, 5170-5177.

Simard, C., Müller, P., Houde, A., Brassard, J. and Trottier, Y.L. (2007) Laboratory procedure: Concentration of hepatitis A virus and rotavirus in spring or mineral bottled water samples and their detection by the reverse-transcriptase polymerase chain reaction. Ottawa, Canada: Health Canada.

Simmons, F.J. and Xagoraraki, I. (2011) Release of infectious human enteric viruses by full-scale wastewater utilities. *Water Research* **45**, 3590-3598.

Singh, N., Singh, R.K., Bhunia, A.K. and Stroshine, R.L. (2002) Effect of inoculation and washing methods on the efficacy of different sanitizers against Escherichia coli O157 : H7 on lettuce. *Food Microbiology* **19**, 183-193.

Skraber, S., Gassiloud, B. and Gantzer, C. (2004) Comparison of coliforms and coliphages as tools for assessment of viral contamination in river water. *Applied and Environmental Microbiology* **70**, 3644-3649.

Sobsey, M.D. and Glass, J.S. (1984) Influence of water-quality on enteric virus concentration by microporous filter methods. *Applied and Environmental Microbiology* **47**, 956-960.

Soerjomataram, I., Oomen, D., Lemmens, V., Oenema, A., Benetou, V., Trichopoulo, A., Coebergh, J. W., Barendregt, J., and de Vries, E. (2010) Increased consumption of fruit and vegetables and future cancer indicende in selected European countries. *European Journal of Cancer* **46**, 2563-2580.

Solomon, E.B. and Matthews, K.R. (2006) Interaction of live and dead *Escherichia coli* O157 : H7 and fluorescent microspheres with lettuce tissue suggests bacterial processes do not mediate adherence. *Letters in Applied Microbiology* **42**, 88-93.

Son, H., Jeong, H.S., Cho, M., Lee, J., Lee, H., Yoon, K., Jeong, A.Y., Jung, S., Kim, K. and Cheon, D.S. (2013) Seroepidemiology of predominant norovirus strains circulating in Korea by using recombinant virus-like particle antigens. *Foodborne Pathogens and Disease* **10**, 461-466.

Song, I., Stine, S.W., Choi, C.Y. and Gerba, C.P. (2006) Comparison of crop contamination by microorganisms during subsurface drip and furrow irrigation. *Journal of Environmental Engineering-Asce* **132**, 1243-1248.

Soto-Beltran, M., Ikner, L.A. and Bright, K.R. (2013) Effectiveness of poliovirus concentration and recovery from treated wastewater by two electropositive filter methods. *Food and Environmental Virology* **5**, 91-96.

Staley, C., Gordon, K.V., Schoen, M.E. and Harwood, V.J. (2012) Performance of two quantitative PCR methods for microbial source tracking of human sewage and implications for microbial risk assessment in recreational waters. *Applied and Environmental Microbiology* **78**, 7317-7326.

Stals, A. (2011) Molecular detection of noroviruses in ready-to-eat foods and fruit products. PhD thesis, Ghent University, Belgium.

Stals, A., Baert, L., Botteldoorn, N., Werbrouck, H., Herman, L., Uyttendaele, M. and Van Coillie, E. (2009a) Multiplex real-time RT-PCR for simultaneous detection of GI/GII noroviruses and murine norovirus 1. *Journal of Virological Methods* **161**, 247-253.

Stals, A., Baert, L., De Keuckelaere, A., Van Coillie, E. and Uyttendaele, M. (2011a) Evaluation of a norovirus detection methodology for ready-to-eat foods. *International Journal of Food Microbiology* **145**, 420-425.

Stals, A., Baert, L., Jasson, V., Van Coillie, E. and Uyttendaele, M. (2011b) Screening of fruit products for norovirus and the difficulty of interpreting positive PCR results. *Journal of Food Protection* **74**, 425-431.

Stals, A., Baert, L., Van Coillie, E. and Uyttendaele, M. (2011c) Evaluation of a norovirus detection methodology for soft red fruits. *Food Microbiology* **28**, 52-58.

Stals, A., Baert, L., Van Coillie, E. and Uyttendaele, M. (2012a) Extraction of food-borne viruses from food samples: A review. *International Journal of Food Microbiology* **153**, 1-9.

Stals, A., Mathijs, E., Baert, L., Botteldoorn, N., Denayer, S., Mauroy, A., Scipioni, A., Daube, G., Dierick, K., Herman, L., Van Coillie, E., Thiry, E. and Uyttendaele, M. (2012b) Molecular detection and genotyping of noroviruses. *Food and Environmental Virology* **4**, 153-167.

Stals, A., Uyttendaele, M., Baert, L. and Van Coillie, E. (2013a) Norovirus transfer between foods and food contact materials. *Journal of Food Protection* **76**, 1202-1209.

Stals, A., Van Coillie, E. and Uyttendaele, M. (2013b) Viral genes everywhere: public health implications of PCR-based testing of foods. *Current Opinion in Virology* **3**, 69-73.

Stals, A., Werbrouck, H., Baert, L., Botteldoorn, N., Herman, L., Uyttendaele, M. and Van Coillie, E. (2009b) Laboratory efforts to eliminate contamination problems in the real-time RT-PCR detection of noroviruses. *Journal of Microbiological Methods* **77**, 72-76.

Steele, M. and Odumeru, J. (2004) Irrigation water as source of foodborne pathogens on fruit and vegetables. *Journal of Food Protection* **67**, 2839-2849.

Steinmann, J., Becker, B., Bischoff, B., Paulmann, D., Friesland, M., Pietschmann, T., Steinmann, J. and Steinmann, E. (2010) Virucidal activity of 2 alcohol-based formulations proposed as hand rubs by the World Health Organization. *American Journal of Infection Control* **38**, 66-68.

Steyer, A., Torkar, K.G., Gutierrez-Aguirre, I. and Poljsak-Prijatelj, M. (2011) High prevalence of enteric viruses in untreated individual drinking water sources and surface water in Slovenia. *International Journal of Hygiene and Environmental Health* **214**, 392-398.

Stine, S.W., Song, I., Choi, C.Y. and Gerba, C.P. (2005a) Effect of relative humidity on preharvest survival of bacterial and viral pathogens on the surface of cantaloupe, lettuce, and bell peppers. *Journal of Food Protection* **68**, 1352-1358.

Stine, S.W., Song, I., Choi, C.Y. and Gerba, C.P. (2011) Application of pesticide sprays to fresh produce: A risk assessment for hepatitis A and *Salmonella*. *Food and Environmental Virology* **3**, 86-91.

Stine, S.W., Song, I.H., Choi, C.Y. and Gerba, C.P. (2005b) Application of microbial risk assessment to the development of standards for enteric pathogens in water used to irrigate fresh produce. *Journal of Food Protection* **68**, 913-918.

Su, X.W. and D'Souza, D.H. (2011) Trisodium phosphate for foodborne virus reduction on produce. *Foodborne Pathogens and Disease* **8**, 713-717.

Su, X.W. and D'Souza, D.H. (2013) Grape seed extract for foodborne virus reduction on produce. *Food Microbiology* **34**, 1-6.

Sukhrie, F.H., Teunis, P., Vennema, H., Copra, C., Beersma, M.F., Bogerman, J. and Koopmans, M. (2012) Nosocomial transmission of norovirus is mainly caused by symptomatic cases. *Clinical Infectious Diseases* **54**, 931-937.

Swinkels, H.M., Kuo, M., Embree, G., Andonov, A., Henry, B., Buxton, J.A. and Fraser Hlth Environm Hlth Invest, T. (2014) Hepatitis A outbreak in British Columbia, Canada: the roles of established surveillance, consumer loyalty cards and collaboration, February to May 2012. *Eurosurveillance* **19**, 36-42.

Symonds, E.M., Griffin, D.W. and Breitbart, M. (2009) Eukaryotic viruses in wastewater samples from the United States. *Applied and Environmental Microbiology* **75**, 1402-1409.

Takahashi, H., Ohuchi, A., Miya, S., Izawa, Y. and Kimura, B. (2011) Effect of food residues on norovirus survival on stainless steel surfaces. *Plos One* **6**.

Takeuchi, K., Matute, C.M., Hassan, A.N. and Frank, J.F. (2000) Comparison of the attachment of *Escherichia coli* O157 : H7, *Listeria monocytogenes*, *Salmonella typhimurium*, and *Pseudomonas fluorescens* to lettuce leaves. *Journal of Food Protection* **63**, 1433-1437.

Taku, A., Gulati, B.R., Allwood, P.B., Palazzi, K., Hedberg, C.W. and Goyal, S.M. (2002) Concentration and detection of caliciviruses from food contact surfaces. *Journal of Food Protection* **65**, 999-1004.

Tam, C.C., Larose, T. and O'Brien, S. (2014) Costed extension to the second study of infectious intestinal disease in the community: identifying the proportion of foodborne disease in the UK and

attributing foodborne disease by food commodity. Project B18021 (FS231043) UK Food Standard Agency, 171 p.

Tamada, Y., Yano, K., Yatsuhashi, H., Inoue, O., Mawatari, F. and Ishibashi, H. (2004) Consumption of wild boar linked to cases of hepatitis E. *Journal of Hepatology* **40**, 869-870.

Tanaka, H., Asano, T., Schroeder, E.D., and Tchobanoglous, G. (1998) Estimating the safety of wastewater reclamation and reuse using enteric virus monitoring data. *Water Environment Research* **70**, 39-51.

Task Force Gastroenteritis (2012) Activity report by the Task Force on Food and Feed Safety in identifying the food-related sources of the gastroenteritis outbreak in Germany. Braunschweig: Federal Office of Consumer Protection and Food Safety (BVL). *Available at: http://wwwbvlbundde/SharedDocs/Downloads/01_Lebensmittel/Task_Force/Task_Force_Gastroenteritis_Activity_Reportpdf;jsessionid=1091D19B658718ECE118673D7AAFA8C81_cid350?__blob=publicationFile&v=2*.

Teltsch, B., Kedmi, S., Bonnet, L., Borenzstajnrotem, Y. and Katzenelson, E. (1980) Isolation and identification of pathogenic microorganisms at wastewater-irrgated fields – ratios in air and wastewater. *Applied and Environmental Microbiology* **39**, 1183-1190.

Teunis, P.F.M. and Havelaar, A.H. (2000) The Beta Poisson dose-response model is not a single-hit model. *Risk Analysis* **20**, 513-520.

Teunis, P. F., Chappell, C. L., and Okhuysen, P. C. (2002a) *Cryptosporidium* dose-response studies: variation between hosts. *Risk Analysis* **22**, 475-485.

Teunis, P. F., Chappell, C. L., and Okhuysen, P. C. (2002b) *Cryptosporidium* dose response studies: variation between isolates. *Risk Analysis* **22**, 175-183.

Teunis, P.F.M., Moe, C.L., Liu, P., Miller, S.E., Lindesmith, L., Baric, R.S., Le Pendu, J. and Calderon, R.L. (2008) Norwalk virus: How infectious is it? *Journal of Medical Virology* **80**, 1468-1476.

Thurnston-Enriquez, J. A., Watt, P., Dowd, S. E., Enriquez, J., Pepper, I. L., Gerba, C. P. (2002) Detection of protozoan parasites and microsporidia in irrigation waters used for crop production. *Journal of Food Protection* **65**, 378-382.

Tian, P., Yang, D. and Mandrell, R. (2011) A simple method to recover Norovirus from fresh produce with large sample size by using histo-blood group antigen-conjugated to magnetic beads in a recirculating affinity magnetic separation system (RCAMS). *International Journal of Food Microbiology* **147**, 223-227.

Tierney, J.T., Sullivan, R. and Larkin, E.P. (1977) Persistence of poliovirus 1 in soil and on vegetables grown in soil previously flooded with inoculated sewage sludge or effluent. *Applied and Environmental Microbiology* **33**, 109-113.

Todd, E.C.D., Michaels, B.S., Holah, J., Smith, D., Greig, J.D. and Bartleson, C.A. (2010) Outbreaks where food workers have been implicated in the spread of foodborne disease. Part 10. Alcohol-based antiseptics for hand disinfection and a comparison of their effectiveness with soaps. *Journal of Food Protection* **73**, 2128-2140.

Topping, J.R., Schnerr, H., Haines, J., Scott, M., Carter, M.J.,Willcock, M.M., Bellamy, K., Brown, D.W., Gary, J.J., Gallimore, C.I. and Knight, A.I. (2009) Temperature inactivation of feline calicivirus vaccine strain FCV F-9 in comparison with human noroviruses using an RNA exposure assay and

reverse transcribed qunatitative real-time polymerase chain reaction – A novel method for predicting virus infectivity. *Journal of Virological Methods* **156**, 89-95.

Trivedi, T.K., Desai, R., Hall, A.J., Patel, M., Parashar, U.D. and Lopman, B.A. (2013) Clinical characteristics of norovirus-associated deaths: A systematic literature review. *American Journal of Infection Control* **41**, 654-657.

Tuladhar, E., Hazeleger, W.C., Koopmans, M., Zwietering, M.H., Beumer, R.R. and Duizer, E. (2012) Residual viral and bacterial contamination of surfaces after cleaning and disinfection. *Applied and Environmental Microbiology* **78**, 7769-7775.

Turcios, R.M., Widdowson, M.A., Sulka, A.C., Mead, P.S. and Glass, R.I. (2006) Reevaluation of epidemiological criteria for identifying outbreaks of acute gastroenteritis due to norovirus: United States, 1998-2000. *Clinical Infectious Diseases* **42**, 964-969.

US EPA (1989) National primary drinking water regulations: filtration, disinfection, turbidity, Giardia lamblia, viruses, Legionella and heterotrophic bacteria; final rule (40 CFR Parts 141 and 142). Fed. Regist. 54:27486.

US EPA (2006) Ultraviolet disinfection guidance manual for the final long term 2 enhanced surface water treatment rule. EPA 815-R-06-007 Washington, DC: *US Environmental Protection Agency*.

US EPA (2011) Problem formulation for human health risk assessment of pathogens in land-applied biosolids. *National Center for Environmental Assessment*, Cincinnati, OH EPA/600/R-08/035F.

Ueki, Y., Sano, D., Watanabe, T., Akiyama, K. and Omura, T. (2005) Norovirus pathway in water environment estimated by genetic analysis of strains from patients of gastroenteritis, sewage, treated wastewater, river water and oysters. *Water Research* **39**, 4271-4280.

van Ginneken, M. and Oron, G. (2000) Risk assessment of consuming agricultural products irrigated with reclaimed wastewater: An exposure model. *Water Resources Research* **36**, 2691-2699.

Van Haute, S. (2014) Strategies for disinfection of fresh produce wash water. Doctoral dissertation, Ghent University, Belgium.

Van Haute, S., Sampers, I., Holvoet, K. and Uyttendaele, M. (2013a) Physicochemical quality and chemical safety of chlorine as a reconditioning agent and wash water disinfectant for fresh-cut lettuce washing. *Applied and Environmental Microbiology* **79**, 2850-2861.

Van Haute, S., Sampers, I., Jacxsens, L. and Uyttendaele, M. (2013b) Selection criteria for water disinfection techniques in agricultural practices. *Critical Reviews in Food Science and Nutrition, DOI: 101080/104083982012705360*.

van Zyl, W.B., Page, N.A., Grabow, W.O.K., Steele, A.D. and Taylor, M.B. (2006) Molecular epidemiology of group A rotaviruses in water sources and selected raw vegetables in southern Africa. *Applied and Environmental Microbiology* **72**, 4554-4560.

Vasickova, P., Dvorska, L., Lorencova, A. and Pavlik, I. (2005) Viruses as a cause of foodborne diseases: a review of the literature. *Veterinarni Medicina* **50**, 89-104.

Vaughan, G., Goncalves Rossi, L. M., Forbi, J. C., de Paula, V. S., Purdy, M. A., Xia, G. and Khudyakov, Y. E. (2014). Hepatitis A virus: host interactions, molecular epidemiology and evolution. *Infection, Genetics and Evolution* **21**, 227-243.

Vega, E., Smith, J., Garland, J., Matos, A. and Pillai, S.D. (2005) Variability of virus attachment patterns to butterhead lettuce. *Journal of Food Protection* **68**, 2112-2117.

Verani, M., Bigazzi, R. and Carducci, A. (2014) Viral contamination of aerosol and surfaces through toilet use in health care and other settings. *American Journal of Infection Control* **42**, 758-762.

Verhaelen, K., Bouwknegt, M., Carratalà, A., Lodder-Verschoor, F., Diez-Valcarce, M., Rodriguez-Lazaro, D., de Roda Husman, A.M. and Rutjes, S.A. (2013a) Virus transfer proportions between gloved fingertips, soft berries, and lettuce, and associated health risks. *International Journal of Food Microbiology* **166**, 419-425.

Verhaelen, K., Bouwknegt, M., Lodder-Verschoor, F., Rutjes, S.A. and de Roda Husman, A.M. (2012) Persistence of human norovirus GII.4 and GI.4, murine norovirus, and human adenovirus on soft berries as compared with PBS at commonly applied storage conditions. *International Journal of Food Microbiology* **160**, 137-144.

Verhaelen, K., Bouwknegt, M., Rutjes, S.A. and Husman, A.M.D. (2013b) Persistence of human norovirus in reconstituted pesticides Pesticide application as a possible source of viruses in fresh produce chains. *International Journal of Food Microbiology* **160**, 323-328.

Verhoef, L. (2011) Identifying international foodborne norovirus outbreak events - A combined epidemiological and molecular approach: PhD dissertation, National Institute for Public Health and the Environment (RIVW), The Netherlands.

Verhoef, L., Gutierrez, G.J., Koopmans, M. and Boxman, I.L.A. (2013) Reported behavior, knowledge and awareness toward the potential for norovirus transmission by food handlers in Dutch catering companies and institutional settings in relation to the prevalence of norovirus. *Food Control* **34**, 420-427.

Verhoef, L., Kouyos, R.D., Vennema, H., Kroneman, A., Siebenga, J., van Pelt, W., Koopmans, M. and Food Borne Viruses Europe Network (2011) An integrated approach to identifying international foodborne norovirus outbreaks. *Emerging Infectious Diseases* **17**, 412-418.

Verhoef, L., Vennema, H., van Pelt, W., Lees, D., Boshuizen, H., Henshilwood, K., Koopmans, M. and Food-Borne Viruses Europe Network (2010) Use of norovirus genotype profiles to differentiate origins of foodborne outbreaks. *Emerging Infectious Diseases* **16**, 617-624.

Verhoef, L.P.B., Kroneman, A., van Duynhoven, Y., Boshuizen, H., van Pelt, W., Koopmans, M. and Foodborne Viruses Europe Network (2009) Selection tool for foodborne norovirus outbreaks. *Emerging Infectious Diseases* **15**, 31-38.

Viau, E., Bibby, K., Paez-Rubio, T. and Peccia, J. (2011) Toward a consensus view on the infectious risks associated with land application of sewage sludge. *Environmental Science & Technology* **45**, 5459-5469.

Victoria, M., Guimaraes, F., Fumian, T., Ferreira, F., Vieira, C., Leite, J.P. and Miagostovich, M. (2009) Evaluation of an adsorption-elution method for detection of astrovirus and norovirus in environmental waters. *Journal of Virological Methods* **156**, 73-76.

Victoria, M., Rigotto, C., Moresco, V., Correa, A.D., Kolesnikovas, C., Leite, J.P.G., Miagostovich, M.P. and Barardi, C.R.M. (2010) Assessment of norovirus contamination in environmental samples from Florianopolis City, Southern Brazil. *Journal of Applied Microbiology* **109**, 231-238.

Villar, L.M., de Paula, V.S., Diniz-Mendes, L., Lampe, E. and Gaspar, A.M.C. (2006) Evaluation of methods used to concentrate and detect hepatitis A virus in water samples. *Journal of Virological Methods* **137**, 169-176.

Vinci, R., Jacxsens, L., Van Loco, J., Matsiko, E., Lachat, C., de Schaetzen, T., Canfyn, M., Van Overmeire, I., Kolsteren, P., and De Meulenaer, B. (2012) Assessment of human exposure to benzene through foods from the Belgian market. *Chemosphere* **88**, 1001-1007.

Vivancos, R., Shroufi, A., Sillis, M., Aird, H., Gallimore, C.I., Myers, L., Mahgoub, H. and Nair, P. (2009) Food-related norovirus outbreak among people attending two barbeques: epidemiological, virological, and environmental investigation. *International Journal of Infectious Diseases* **13**, 629-635.

VMM (2005) Water elke druppel telt. Aardappel-, fruit- en groenteverwerkende industrie. http://www.vlaanderen.be/nl/publicaties/detail/water-elke-druppel-telt-aardappel-fruit-en-groenteverwerkende-industrie

Vose, D. (2008) Risk analysis: a quantitative guide. West Sussex, England: John Wiley & Sons.

Wachtel, M.R. and Charkowski, A.O. (2002) Cross-contamination of lettuce with *Escherichia coli* O157 : H7. *Journal of Food Protection* **65**, 465-470.

Wadl, M., Scherer, K., Nielsen, S., Diedrich, S., Ellerbroek, L., Frank, C., Gatzer, R., Hoehne, M., Johne, R., Klein, G., Koch, J., Schulenburg, J., Thielbein, U., Stark, K. and Bernard, H. (2010) Food-borne norovirus-outbreak at a military base, Germany, 2009. *Bmc Infectious Diseases* **10**.

Wallis, C., Melnick, J.L. and Gerba, C.P. (1979) Concentration of viruses from water by membrane chromatography. *Annual Review of Microbiology* **33**, 413-437.

Wang, Q., Erickson, M., Ortega, Y.R. and Cannon, J.L. (2012) The fate of murine norovirus and hepatitis A virus during preparation of fresh produce by cutting and grating. *Food Environmental Virology* **5**, 52-60.

Wang, Q., Erickson, M.C., Ortega, Y. and Cannon, J.L. (2013a) Physical removal and transfer of murine norovirus and hepatitis A virus from contaminated produce by scrubbing and peeling. *Journal of Food Protection* **76**, 85-92.

Wang, Q., Hirneisen, K.A., Markland, S.M. and Kniel, K.E. (2013b) Survival of murine norovirus, tulane virus, and hepatitis A virus on alfalfa seeds and sprouts during storage and germination. *Applied and Environmental Microbiology* **79**, 7021-7027.

Wang, Q.H., Han, M.G., Cheetham, S., Souza, M., Funk, J.A. and Saif, L.J. (2005) Porcine noroviruses related to human noroviruses. *Emerging Infectious Diseases* **11**, 1874-1881.

Wang, X., Young, O.A. and Karl, D.P. (2010) Evaluation of cleaning procedures for allergen control in a food industry environment. *Journal of Food Science* **75**, T149-T155.

Warner, R.D., Carr, R.W., McCleskey, F.K., Johnson, P.C., Elmer, L.M.G. and Davison, V.E. (1991) A large nontypical outbreak of norwalk virus – gastroenteritis associated with exposing celery to nonpotable water and with *Citrobacter-freundii*. *Archives of Internal Medicine* **151**, 2419-2424.

Wei, J., Jin, Y., Sims, T. and Kniel, K.E. (2010) Manure- and biosolids-resident murine norovirus 1 attachment to and internalization by romaine lettuce. *Applied and Environmental Microbiology* **76**, 578-583.

Wei, J., Jin, Y., Sims, T. and Kniel, K.E. (2011) Internalization of murine norovirus 1 by *Lactuca sativa* during irrigation. *Applied and Environmental Microbiology* **77**, 2508-2512.

Wei, J. and Kniel, K.E. (2010) Pre-harvest viral contamination of crops originating from fecal matter. *Food and Environmental Virology* **2**, 195-206.

Wendel, A.M., Johnson, D.H., Sharapov, U., Grant, J., Archer, J.R., Monson, T., Koschmann, C. and Davis, J.P. (2009) Multistate outbreak of *Escherichia coli* O157:H7 infection associated with consumption of packaged spinach, August-September 2006: The Wisconsin Investigation. *Clinical Infectious Diseases* **48**, 1079-1086.

Westrell, T., Teunis, P., van den Berg, H., Lodder, W., Ketelaars, H., Stenstrom, T.A. and Husman, A.M.D. (2006) Short- and long-term variations of norovirus concentrations in the Meuse river during a 2-year study period. *Water Research* **40**, 2613-2620.

Wheeler, C., Vogt, T.M., Armstrong, G.L., Vaughan, G., Weltman, A., Nainan, O.V., Dato, V., Xia, G.L., Waller, K., Amon, J., Lee, T.M., Highbaugh-Battle, A., Hembree, C., Evenson, S., Ruta, M.A., Williams, I.T., Fiore, A.E. and Bell, B.P. (2005) An outbreak of hepatitis A associated with green onions. *New England Journal of Medicine* **353**, 890-897.

Who (2004) Guidelines for drinking-water quality (Third edition). *World Health Organisation, Geneva*, ISBN 978924154761 978924154761 download from http://www.who.int/water_sanitation_health/dwq/gdwaq978924154763/en.

Who (2006) Guidelines for the safe use of wastewater, excreta and greywater-volume 2: wastewater use in agriculture. *World Health Organisation, Geneva*.

WHO (2014) http://www.who.int/csr/disease/hepatitis/whocdscsredc2007/en/index4.html.

Wigginton, K.R., Pecson, B., Sigstam, T., Bosshard, F. and Kohn, T. (2012) Virus inactivation mechanisms: impact of disinfectants on virus function and structural integrity. *Environmental Science & Technology* **46**, 12069-12078.

Wilkes, G., Edge, T., Gannon, V., Jokinen, C., Lyautey, E., Medeiros, D., Neumann, N., Ruecker, N., Topp, E. and Lapen, D.R. (2009) Seasonal relationships among indicator bacteria, pathogenic bacteria, *Cryptosporidium* oocysts, *Giardia* cysts, and hydrological indices for surface waters within an agricultural landscape. *Water Research* **43**, 2209-2223.

Williams-Woods, J., Gonzalez-Escalona, N. and Burkhardt, W. (2011) Direct sequencing of hepatitis A virus and norovirus RT-PCR products from environmentally contaminated oyster using M13-tailed primers. *Journal of Virological Methods* **178**, 253-257.

Wobus, C.E., Karst, S.M., Thackray, L.B., Chang, K.O., Sosnovtsev, S.V., Belliot, G., Krug, A., Mackenzie, J.M., Green, K.Y. and Virgin, H.W. (2004) Replication of Norovirus in cell culture reveals a tropism for dendritic cells and macrophages. *Plos Biology* **2**, 2076-2084.

Won, G., Kline, T.R. and LeJeune, J.T. (2013) Spatial-temporal variations of microbial water quality in surface reservoirs and canals used for irrigation. *Agricultural Water Management* **116**, 73-78.

Wu, H.M., Fornek, M., Schwab, K.J., Chapin, A.R., Gibson, K., Schwab, E., Spencer, C. and Henning, K. (2005) A norovirus outbreak at a long-term-care facility: The role of environmental surface contamination. *Infection Control and Hospital Epidemiology* **26**, 802-810.

Wu, J., Rodriguez, R.A., Stewart, J.R. and Sobsey, M.D. (2011) A simple and novel method for recovering adenovirus 41 in small volumes of source water. *Journal of Applied Microbiology* **110**, 1332-1340.

Wyn-Jones, A.P., Carducci, A., Cook, N., D'Agostino, M., Divizia, M., Fleischer, J., Gantzer, C., Gawler, A., Girones, R., Holler, C., Husman, A.M.D., Kay, D., Kozyra, I., Lopez-Pila, J., Muscillo, M., Nascimento, M.S., Papageorgiou, G., Rutjes, S., Sellwood, J., Szewzyk, R. and Wyer, M. (2011) Surveillance of adenoviruses and noroviruses in European recreational waters. *Water Research* **45**, 1025-1038.

Wyn-Jones, A.P., Pallin, R., Dedoussis, C., Shore, J. and Sellwood, J. (2000) The detection of small round-structured viruses in water and environmental materials. *Journal of Virological Methods* **87**, 99-107.

Wyn-Jones, A.P. and Sellwood, J. (2001) Enteric viruses in the aquatic environment. *Journal of Applied Microbiology* **91**, 945-962.

Xi, J.A., Graham, D.Y., Wang, K.N. and Estes, M.K. (1990) Norwalk virus genome cloning and characterization. *Science* **250**, 1580-1583.

Xie, Y., Hajdok, C., Mittal, G.S. and Warriner, K. (2008) Inactivation of MS2 F(+) coliphage on lettuce by a combination of UV light and hydrogen peroxide. *Journal of Food Protection* **71**, 903-907.

Yamashita, T., Kobayashi, S., Sakae, K., Nakata, S., Chiba, S., Ishihara, Y. and Isomura, S. (1991) Isolation of cytopathic small round viruses with BS-C-1 cells from patients with gastroenteritis. *Journal of Infectious Diseases* **164**, 954-957.

Yamashita, Y., Ootsuka, Y., Kondo, R., Oseto, M., Doi, M., Miyamoto, T., Ueda, T., Kondo, H., Tanaka, T., Wakita, T., Katayama, K., Takeda, N. and Oka, T. (2010) Molecular characterization of sapovirus detected in a gastroenteritis outbreak at a wedding hall. *Journal of Medical Virology* **82**, 720-726.

Yang, Y.S., Meier, F., Lo, J.A., Yuan, W.Q., Sze, V.L.P., Chung, H.J. and Yuk, H.G. (2013) Overview of recent events in the microbiological safety of sprouts and new intervention technologies. *Comprehensive Reviews in Food Science and Food Safety* **12**, 265-280.

Yepiz-Gomez, M.S., Gerba, C.P. and Bright, K.R. (2013) Survival of respiratory viruses on fresh produce. *Food and Environmental Virology* **5**, 150-156.

Yilmaz, A., Bostan, K., Altan, E., Muratoglu, K., Turan, N., Tan, D., Helps, C. and Yilmaz, H. (2011) Investigations on the frequency of norovirus contamination of ready-to-eat food items in Istanbul, Turkey, by using real-time reverse transcription PCR. *Journal of Food Protection* **74**, 840-843.

Yu, J.H., Kim, N.Y., Koh, Y.J. and Lee, H.J. (2010) Epidemiology of foodborne norovirus outbreak in Incheon, Korea. *Journal of Korean Medical Science* **25**, 1128-1133.

Zeng, W., Vorst, K., Brown, W., Marks, B.P., Jeong, S., Pérez-Rodriguez, F. and Ryser, E.T. (2014) Growth of *Escherichia coli* O157:H7 and *Listeria monocytogenes* in packaged fresh-cut romaine mix at fluctuating temperatures during commercial transport, retail storage, and display. *Journal of Food Protection* **77**, 197-206.

Zhang, G.D., Ma, L., Phelan, V.H. and Doyle, M.P. (2009) Efficacy of antimicrobial agents in lettuce leaf processing water for control of *Escherichia coli* O157:H7. *Journal of Food Protection* **72**, 1392-1397.

Zheng, D.P., Ando, T., Fankhauser, R.L., Beard, R.S., Glass, R.I. and Monroe, S.S. (2006) Norovirus classification and proposed strain nomenclature. *Virology* **346**, 312-323.

Zhou, F.H., Harmon, K.M., Yoon, K.J., Olson, D.G. and Dickson, J.S. (2011) Inactivation of feline calicivirus as a surrogate for norovirus on lettuce by electron beam irradiation. *Journal of Food Protection* **74**, 1500-1503.

Zomer, T.P., de Jong, B., Kuhlmann-Berenzon, S., Nyren, O., Svenungsson, B., Hedlund, K.O., Ancker, C., Wahl, T. and Andersson, Y. (2010) A foodborne norovirus outbreak at a manufacturing company. *Epidemiology and Infection* **138**, 501-506.

Zuber, S., Butot, S. and Baert, L. (2013) Effects of treatments used in food processing on viruses. In *Foodborne viruses and prions and their significance for public health* eds. Smulders, F.J.M., Norrung, B. and Budka, H.: Wageningen Academic Publishers.

CURRICULUM VITAE

CURRICULUM VITAE

Ann De Keuckelaere was born in Ghent on the 12th of April 1987. In 2005 she finished high school, and five years later she graduated as a Master of Science in Bioscience Engineering: Food Science and Nutrition at Ghent University.

Her master thesis, concerning the detection of noroviruses in food and water, was completed at the Lab for Food Microbiology and Food Preservation (Faculty of Bioscience Engineering). In August 2010, she started as a PhD researcher at the same lab under the guidance of Prof. dr. ir. Mieke Uyttendaele as promotor. The first five months (August-December 2010) as a PhD researcher on the European project Veg-i-Trade and from the start of January 2011 on her own IWT PhD scholarship.

During this research she participated in various national and international conferences, published in international peer reviewed journals, and was active as a reviewer for Food Research International. Furthermore, she guided several students and assisted in the practical sessions of the Ghent University course of Molecular Microbial Techniques and Food Microbiology and Food Preservation. In the framework of this PhD research, she also cooperated with some processing companies for sampling of frozen raspberries and discussion on norovirus results.

CURRICULUM VITAE

Ann De Keuckelaere werd geboren op 12 april 1987 te Gent. In 2005 behaalde zij het diploma wetenschappen-wiskunde aan het Emmaüs instituut te Aalter, vijf jaar later promoveerde zij tot Bio-ingenieur in de Levensmiddelenwetenschappen en Voeding aan de Universiteit Gent.

De masterproef, omtrent detectie van norovirussen in levensmiddelen en water, werd volbracht aan het Labo voor Levensmiddelenmicrobiologie en –conservering (Faculteit Bio-ingenieurswetenschappen). In augustus 2010, startte zij als doctoraatsbursaal op hetzelfde labo onder het promotorschap van Prof. dr. ir. Mieke Uyttendaele. De eerste vijf maand (augustus-december 2010) als onderzoeker op het Europees project Veg-i-Trade en vanaf januari 2011 als IWT bursaal met een persoonsgebonden doctoraatsbeurs.

Tijdens dit onderzoek nam zij deel aan verscheidene nationale en internationale conferenties, publiceerde zij artikels in internationale tijdschriften en was actief als reviewer voor Food Research International. Tevens begeleidde zij verscheidene studenten en werkte mee aan de practica voor de vakken “Moleculair-microbiële technieken”, en “Levensmiddelenmicrobiologie en -conservering” in de opleiding Bio-ingenieur. Gedurende dit doctoraatsonderzoek, werd tevens overleg gepleegd met een aantal bedrijven met betrekking tot monstername en interpretatie van norovirus resultaten op bevroren frambozen.

Publications in A1 peer-reviewed journals

De Keuckelaere, A., Jacxsens, L., Amoah, P., Medema, G. J., McClure, P., Jaykus, L.-A., Uyttendaele, M. (under review). Zero risk does not exist: Lessons learned from microbial risk assessment related to use water and safety of fresh produce. Comprehensive Reviews in Food Science and Food Safety.

De Keuckelaere, A., Li, D., Deliens, B., Stals, A., Uyttendaele, M., 2015. Batch testing for noroviruses in frozen raspberries. International Journal of Food Microbiology 192, 43-50.

Li, D., **De Keuckelaere, A.**, Uyttendaele, M., 2014. Application of long-range and binding reverse transcription-quantitative PCR to indicate the viral integrities of noroviruses. Applied and Environmental Microbiology 80, 6473-6479.

De Keuckelaere, A., Stals, A., Uyttendaele, M., 2014. Semi-direct lysis of swabs and evaluation of their efficiencies to recover human noroviruses GI and GII from surfaces. Food and Environmental Virology 6, 132-139.

Holvoet, K., **De Keuckelaere, A.** (co-first author), Sampers, I., Van Haute, S., Stals, A., Uyttendaele, M., 2014. Quantitative study of cross-contamination with *Escherichia coli*, *E. coli* O157, MS2 phage and murine norovirus in a simulated fresh-cut lettuce wash process. Food Control 37, 218-227.

De Keuckelaere, A., Stals, A., Baert, L., Uyttendaele, M., 2013. Performance of two real-time RT-PCR assays for the quantification of GI and GII noroviruses and hepatitis A virus in environmental water samples. Food Analytical Methods 6, 1016-1023.

De Keuckelaere, A., Baert, L., Duarte, A., Stals, A., Uyttendaele, M., 2013. Evaluation of viral concentration methods from irrigation and processing water. Journal of Virological Methods 187, 294-303.

Stals, A., Baert, L., **De Keuckelaere, A.**, Van Coillie, E., Uyttendaele, M., 2011. Evaluation of a norovirus detection methodology for ready-to-eat foods. International Journal of Food Microbiology 145, 420-425.

Book chapter

Li, D., **De Keuckelaere, A.**, Uyttendaele, M. Impact of food unit operations on virus loads in foods in Quantitative microbiology in food processing – Modeling the effects of processing operations on microbial ecology of foods (Eds. De Souza Sant'Ana, A.), submitted for publication.

Extended abstracts of symposia

Li, D., **De Keuckelaere, A.**, Uyttendaele, M., 2014. Application of binding- and long range-RT-quantitative (Q)PCR to indicate the viral integrities of noroviruses. Oral presentation. 4th Food and Environmental Virology conference, 2-5 September, Corfu, Greece.

Uyttendaele, M., **De Keuckelaere, A.**, Li, D., 2014. Harmonization of methods to detect norovirus in foods and environmental samples. Oral presentation. International Association for Food Protection annual meeting, 3-6 August, Indianapolis, Indiana, United States.

Uyttendaele, M., Stals, A., **De Keuckelaere, A.**, Li, D., 2013. Consideration of the extraction efficiencies in the detection of noroviruses from food and environmental samples. Oral presentation. Fifth International Calicivirus Conference, 12-15 October, Beijing, China.

De Keuckelaere, A., Stals, A., Deliens, B., Uyttendaele, M., 2013. Prevalence study of noroviruses on raspberries. Poster presentation. 18th Conference on Food Microbiology, 12-13 September, Brussels, Belgium.

De Keuckelaere, A., Stals, A., Holvoet, K., Lopez Galvez, F., Li, D., Uyttendaele, M., 2012. Quantification study of cross-contamination of fresh-cut lettuce with viruses during a simulation of an industrial wash process. Oral presentation. 3rd Food and Environmental Virology Conference, 7-10 October, Lisbon, Portugal.

Delbeke, S., Baert, L., **De Keuckelaere, A.**, Li, D., Uyttendaele, M., 2012. Survival of norovirus, murine norovirus 1, MS2 phage and *E. coli* in various types of water used for irrigation of fresh produce. Poster presentation. 17th Conference on Food Microbiology, 20-21 September, Brussels, Belgium.

Delbeke, S., Baert, L., **De Keuckelaere, A.**, Li, D., Uyttendaele, M., 2012. Survival of norovirus, murine norovirus 1, MS2 phage and *E. coli* in various types of water used for irrigation of fresh produce. Oral presentation. IAFP European symposium on food safety, 21-23 May, Warsaw, Poland.

De Keuckelaere, A., Baert, L., Duarte, A. M. M., Delbeke, S., Uyttendaele, M., 2011. Evaluation of viral concentration methods from processing water and different types of irrigation water using murine norovirus-1 and MS2. Poster presentation. 16th Conference on Food Microbiology, 22-23 September, Brussels, Belgium.

De Keuckelaere, A., Baert, L., Duarte, A. M. M., Delbeke, S., Uyttendaele, M., 2011. Evaluation of viral concentration methods from processing water and differetn types of irrigation water using murine norovirus-1 and MS2. Poster presentation. VITAL Conference, 5-7 September, Ljubljana, Slovenia.

De Keuckelaere, A., Baert, L., Stals, A., De Vocht, M., Li, D., Delbeke, S., Lauryssen, S., Jacxsens, L., Sas, B., Uyttendaele, M., 2011. Survey conducted by a consumer organization for the presence of bacterial and viral pathogens on high risk fresh produce from the Belgian market. Oral presentation. 2nd International Conference on Quality Management of Fresh Cut Produce: Convenience for a tasteful life, 17-21 July, Torino, Italy.

De Keuckelaere, A., Baert, L., Stals, A., Li, D., Delbeke, S., Lauryssen, S., Jacxsens, L., Uyttendaele, M., 2011. Survey conducted by a consumer organization for the presence of bacterial and viral pathogens on high risk fresh produce from the Belgian market. Poster presentation. IAFP European symposium on food safety, 18-20 May, Ede, The Netherlands.

De Keuckelaere, A., 2010. Detectie van norovirus in levensmiddelen en water. Poster presentation. ie-prijzen event, 25 November, Brussel, Belgium. *Laureaat ie-prijzen 2010 categorie voeding*.

Stals, A., Baert, L., **De Keuckelaere, A.**, Van Coillie, E., Uyttendaele, M., 2010. Evaluation of a norovirus detection methodology for ready-to-eat foods. Poster presentation. 4th International Conference on Caliciviruses, 16-19 October, Santa Cruz, Chili.

Stals, A., Baert, L., **De Keuckelaere, A.**, Van Coillie, E., Uyttendaele, M., 2010. Evaluation of a norovirus detection methodology for ready-to-eat foods. Oral presentation. 2nd COST 929 symposium, 7-9 October, Istanbul, Turkey.

Stals, A., Baert, L., **De Keuckelaere, A.**, Van Coillie, E., Uyttendaele, M., 2010. Evaluation of a norovirus detection methodology for ready-to-eat foods. Poster presentation. 15th Conference of Food Microbiology, 16-17 September, Brussels, Belgium.

Dissemination

De Keuckelaere, A., Stals, A., Jacxsens, L., Rajkovic, A., Uyttendaele, M., 2014. The issue of norovirus in leafy greens and raspberries. Oral presentation. Veg-i-Trade: 6th consortium meeting, March 21th, Kruger National Park, South Africa.

De Keuckelaere, A., Stals, A., Holvoet, K., Sampers, I., Uyttendaele, M., 2013. Cross-contamination of norovirus during the washing process of lettuce + **De Keuckelaere, A.**, Deliens, B., Stals, A., Uyttendaele, M., 2013. Prevalence study of norovirus on raspberries. Oral presentations. Mini Symposium on virus projects, 13 June, Ghent University, Ghent, Belgium.

De Keuckelaere, A., Baert, L., 2011. Detection of norovirus and F-specific RNA bacteriophages in fresh produce and water. 27 June – 4 July, Veg-i-Trade training session, Norwegian Veterinary Institute, Oslo, Norway.

Doctoral Schools program

Specialist courses

2012	Gene technology and molecular diagnostics (I000366) – Prof. Gheysen, G.
2012	Bfr Summer School on Risk Assessment and Risk Communication August 13-24, Berlin, Germany
2013	Interphase processes of host-associated micro-organisms (I000524) – Prof. Van de Wiele, T.

Personal skills training

2011	Advanced academic English: conference skills – English proficiency for Presentations (A002777)
2011	Project management
2012	Personal effectiveness (X000172)
2013	Advanced academic English: writing skills ((Bioscience) Engineering) (A002774)